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13. ABSTRACT *(Maximum 200 Words)*

During the first year of this award, we have made great progress toward defining the molecular basis for androgen independent prostate cancer progression. A key theme from our findings is the concept that genes which cause androgen independence can do so by activating the androgen receptor in a ligand-independent fashion. Several lines of evidence from our work support this hypothesis. We have identified two genes - the Her2/neu receptor tyrosine kinase and the serine/threonine kinase MEKK1 - which can activate the androgen receptor and have major effects on prostate cancer growth. In the case of Her2/neu, the availability of drugs which can attack the Her2/neu protein raise the possibility of clinical trials to address the role of this gene in human prostate cancer. We have also isolated a third gene, encoding the serine protease cathepsin D (unpublished data), which confers androgen independent growth in prostate cancer cells in SCID mouse models. Current work is focused on developing transgenic mouse models of prostate cancer based on these genes. Specifically, we have generated several founder lines expressing cathepsin D under the control of the prostate-specific probasin promoter and are in the process of characterizing this phenotype. These mice should lead to newer mouse models and will allow us to define the role of this protease in prostate cancer.

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Table of Contents

Introduction

The goal of this research is to identify and characterize genes which are responsible for the progression of prostate cancer to androgen independence. Our strategy is to use a series of human prostate cancer xenografts established by us (LAPC 4 and LAPC 9) to isolate these genes through expression cloning. In the first year of the project we have completed task 1 (isolation of genes) and are currently focused on characterizing the biological characteristics of these genes in our prostate cancer models. After extensive screening we have chosen to focus on three genes - Her2/neu, MEKK1, and cathepsin D - which are described independently below. In each case, the biological characterization includes overexpression of the gene in androgen-dependent prostate cells and examination of the growth properties of those cells in vitro and in SCID mice. We are also examining the effects on signaling through the androgen receptor, as we believe this may be a major mechanism by which these genes cause androgen-independent growth. These points are best illustrated in the preprint describing a role for Her2/neu in prostate cancer (Craft et al, Nature Medicine, 1999). We are also examining the effects of these genes in the context of the mouse prostate gland through the creation of transgenic mice. Taken together, these functional studies using both human prostate cancer xenografts and transgenic mouse models will teach us a great deal about mechanisms of androgen independent progression.

Body

<u>Evidence for clonal evolution as a mechanism for androgen independence based on xenograft studies</u>

Prostate cancers require androgen for growth but progress to an androgen-independent stage under the selective pressure of androgen ablation therapy. In the past year we have characterized a novel prostate cancer xenograft (LAPC-9) propagated by serial passage in male SCID mice that expresses prostate specific antigen and wild-type androgen receptor. In response to castration, LAPC-9 cells undergo growth arrest and persist in a dormant, androgen-responsive state for at least 6 months. After prolonged periods of androgen deprivation, spontaneous androgen-independent outgrowths develop. Thus, prostate cancers progress to androgen-independence through two distinct stages, initially escaping dependence on androgen for survival and, subsequently, for growth. Through the use of serial dilution and fluctuation analysis, we provide evidence that the latter stage of androgen-independence results from clonal expansion of androgen-independent cells which are present at a frequency of about 1 per 10^5-10^6 androgen-dependent cells. We conclude that prostate cancers contain heterogeneous mixtures of cells that vary in their dependence on androgen for growth and survival and that treatment with anti-androgen therapy alters the relative frequency of these cells, thereby leading to outgrowths of androgen-independent cancers. This work is described in detail in the attached reprint in press at <u>Cancer Research</u> (Craft et al, 1999).

<u>Her2/neu activates androgen receptor signaling and causes androgen independent growth.</u>

A key event in the progression of prostate cancer involves conversion from a hormone sensitive, androgen-dependent stage to a hormone refractory, androgen-independent tumor. The androgen receptor pathway continues to function in these androgen-independent tumors despite anti-androgen therapy which reduces ligand concentration to trace levels. In our LAPC-4 mouse xenograft model of human prostate cancer we find that androgen independent sublines express higher levels of the HER-2/neu receptor tyrosine kinase than their androgen-dependent counterparts. Forced overexpression of HER-2/neu in androgen-dependent LNCaP prostate cancer cells allows ligand-independent growth in vitro and accelerates the progression to androgen-independence in vivo. HER-2/neu activates the androgen receptor pathway in the absence of ligand and synergizes with low levels of androgen to superactivate the pathway. These findings demonstrate crosstalk between the HER-2/neu and androgen receptor signal transduction pathways. By modulating the response of the androgen receptor to low doses of androgen, HER-2/neu can restore androgen receptor function to prostate

cancer cells, a finding that bears directly on the clinical progression of prostate cancer. These results are presented in detail in the attached reprint from Nature Medicine (Craft et al, 1999).

The MEKK1 serine/threonine kinase activates the androgen receptor pathway in prostate cancer cells.

Mitogen-activated protein (MAP) kinases phosphorylate the estrogen receptor and activate transcription from estrogen receptor-regulated genes. Based on our findings with Her2/neu, we postulated other potential interactions between the MAP kinase cascade and androgen receptor-mediated gene regulation. Specifically, we have studied the biological effects of mitogen-activated protein kinase kinase kinase (MEKK1) expression in prostate cancer cells which are either androgen-dependent (AD) or androgen-independent (AI) for growth. Our findings demonstrate that expression of constitutively active MEKK1 induces apoptosis in androgen receptor-positive but not androgen receptor-negative prostate cancer cells. Reconstitution of the androgen receptor signaling pathway in androgen receptor-negative prostate cancer cells restores MEKK1-induced apoptosis. Overexpression of androgen receptor in androgen receptor-positive prostate cancer cells enhances MEKK1-induced apoptosis, whereas pharmacologic blockade of the androgen receptor partially inhibits MEKK1-induced apoptosis. MEKK1 also stimulates the transcriptional activity of the androgen receptor in the presence or absence of ligand. These studies demonstrate an unanticipated link between MEKK1and hormone receptor signaling and have implications for the molecular basis of hormone independent prostate cancer growth. A more detailed description can be found in the attached reprint from Molecular and Cellular Biology (Abreu-Martin et al, 1999).

Isolation of the serine protease cathepsin D in a screen for androgen independence genes

To better understand the shift from androgen-dependent to androgen-independent prostate cancer growth at a molecular level, we devised a strategy to identify genes from AI prostate cancer which confer androgen-independent growth by functional expression cloning. Previously, we established a SCID mouse xenograft model which recapitulates progression of advanced stage human prostate cancer to hormone independence. We constructed an amphotropic retrovirus cDNA library from the androgen-independent stage to allow high efficiency gene transfer and stable expression of cDNAs in target cells. Two functional screens were employed to identify genes responsible for prostate cancer progression. NIH3T3 cells transduced with the retroviral library were screened for colony formation in soft agar. Individual colonies were expanded in culture and retroviral inserts were recovered by PCR and sequenced. In a separate screen, androgen dependent LNCaP prostate cancer cells, which form colonies in soft agar only in the presence of androgen, were transduced with the androgen-independent retroviral library. Retroviral inserts from colonies that grew in soft agar in the absence of androgen were isolated directly by PCR and sequenced. To date, more than 20 independent cDNA clones have been isolated from the two screens combined. Cathepsin D, a lysosomal aspartyl protease previously implicated in breast cancer progression, was isolated from both screens independently. Cathepsin D was originally identified as an estrogen responsive gene and is known to function as an oncogene in vitro and in vivo. We find that cathepsin D protein is expressed 3-5 fold higher in androgen-independent xenograft tumors compared to androgen-dependent tumors, providing further evidence that cathepsin D plays a role in androgen-independent prostate cancer growth. We have also shown that overexpression of cathepsin D facilitates the outgrowth of androgen independence clones in the androgen-dependent LNCaP cell line, and we have constructed transgenic mice expressing cathepsin D in the prostate gland. These data demonstrate that genes involved in androgen-independent prostate cancer can be identified by functional expression cloning and implicate cathepsin D in the progression of late stage prostate cancer.

Appendix

Research Accomplishments

1. We have identified the Her2/neu tyrosine kinase as a gene which can cause androgen independence.
2. We have identified the MEKK1 serine/threonine kinase as a gene which can activate the androgen receptor.
3. We have provided evidence for the origin of androgen independence through clonal evolution using xenografts developed by our group.
4. We have created transgenic mice expressing the cathepsin D serine protease specifically in the prostate gland as a model of androgen independence.

Manuscripts (attached)

1. Craft N., Shostak Y., Carey M., Sawyers C.L. (1999) A mechanism for hormone independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. Nature Medicine, 5(3):280-285.
2. Abreu-Martin MT, Chari A, Palladino AA, Craft NA, Sawyers C.L. (1999) Mitogenactivated protein kinase kinase kinase (MEKK1) activates androgen receptor-dependent transcription and apoptosis in prostate cancer. Mol Cell Biol, 19(7):5143-5154.
3. Noah Craft, Chloe Chhor, Chris Tran, Arie Belldegrun, Jean DeKernion, Owen N. Witte, Jonathan Said, Robert E. Reiter, Charles L. Sawyers. (1999) Evidence for clonal outgrowth of androgen-independent prostate cancer cells from androgen-dependent tumors through a two-step process. Cancer Res. In press
4. Craft N. and Sawyers C.L. (1999) Mechanistic concepts in androgen-dependence of prostate cancer. Cancer & Metastasis Rev, 17:421-427.

A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase

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Prostate cancer progresses from a hormone-sensitive, androgen-dependent stage to a hormone-refractory, androgen-independent tumor. The androgen receptor pathway functions in these androgen-independent tumors despite anti-androgen therapy. In our LAPC-4 prostate cancer model, androgen-independent sublines expressed higher levels of the HER-2/neu receptor tyrosine kinase than their androgen-dependent counterparts. Forced overexpression of HER-2/neu in androgen-dependent prostate cancer cells allowed ligand-independent growth. HER-2/neu activated the androgen receptor pathway in the absence of ligand and synergized with low levels of androgen to 'superactivate' the pathway. By modulating the response to low doses of androgen, a tyrosine kinase receptor can restore androgen receptor function to prostate cancer cells, a finding directly related to the clinical progression of prostate cancer.

Prostate cancer begins as an androgen-dependent tumor that undergoes clinical regression in response to pharmacological or surgical strategies that reduce testosterone concentration. Despite this treatment, the cancer eventually regrows as an androgen- or hormone-independent tumor. The molecular basis for hormone independent cancer progression is poorly understood. Most androgen-independent prostate tumors continue to express androgen receptor (AR) as well as the androgen-dependent gene prostate-specific antigen (PSA), which indicates that these cells maintain a functional AR signaling pathway despite castrate levels of testosterone. Recent attention has focused on the hypothesis that AR itself mediates androgen-independent progression.

There are two variations of this hypothesis. One is that amplification of AR or mutation in the hormone binding domain, which occur in 20–30% of androgen-independent prostate tumors[1–3], alter its function. In at least one example, mutation alters the specificity of the ligand binding domain such that the mutant AR can bind and respond to other steroid hormones such as estrogen[4]. An alternative model is that recruitment of non-steroid receptor signal transduction pathways activate AR in the setting of clinical androgen deprivation. The progesterone and estrogen receptors (ER) can be activated by epidermal growth factor[5] (EGF), dopamine[6], insulin-like growth factor 1 (IGF-1)(ref. 7) and cAMP (ref. 8). Activation of ER occurs by phosphorylation at Ser 118 through the mitogen-activated protein kinase pathway[9]. AR can also become activated in a ligand-independent manner by IGF-1, EGF and keratinocyte growth factor[10] (KGF), but the mechanistic details are unknown. The implications of these observations for human disease remain unclear.

HER-2/neu, a member of the EGF family of receptor tyrosine kinases, is overexpressed in 20–30% of human breast and ovarian cancers[11]. Several observations indicate interactions between HER-2/neu and ER signaling in breast cancer. Overexpression of HER-2/neu in breast cancer is inversely correlated with ER levels[12,13] and predicts clinical resistance to the anti-estrogen tamoxifen[14,15]. Forced expression of HER-2/neu induces ER activation, tyrosine phosphorylation and confers estrogen-independent growth[16], indicating that there is cross-talk between HER-2/neu and ER. HER-2/neu may also be involved in prostate cancer. HER-2/neu is normally expressed in prostate epithelial cells[17,18], and the heregulin ligand is expressed in the stroma and basal epithelial cells of the normal prostate gland[19]. In some but not all studies, HER-2/neu is overexpressed and/or amplified at the DNA level in a subset of prostate cancer patients[20,21] and has been associated with shortened survival[22,23]. It is difficult to evaluate the frequency of HER-2/neu abnormalities in advanced prostate cancer, as these tissues are not routinely biopsied. However, elevated serum levels of Her2 extracellular domain have been correlated with hormone-refractory disease after endocrine therapy[24].

We have established androgen-dependent human prostate cancer xenografts and developed androgen-independent sublines[25]. During studies of differential gene expression between androgen-dependent and androgen-independent sublines, we noted a consistent increase in HER-2/neu protein levels in association with progression to androgen-independence in the LAPC-4 line. Forced overexpression of HER-2/neu in androgen-dependent prostate cancer cells was sufficient to confer androgen-independent growth *in vitro* and accelerate progression to androgen-independence in castrate animals. HER-2/neu activated the AR signaling pathway in the absence of ligand and enhanced the magnitude of AR response in the presence of low levels of androgen. Reconstitution experiments established that the effects of HER-2/neu on the AR pathway require expression of AR. These findings demonstrate that there is cross-talk between the HER-2/neu and AR pathways, and provide mechanistic insight into the clinical problem of androgen-independent prostate cancer progression.

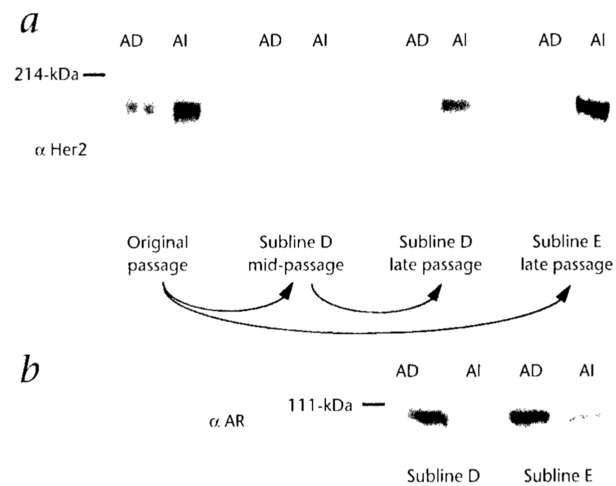


Fig. 1 Expression of HER-2/neu in androgen-dependent and androgen-independent sublines of human prostate cancer xenografts. Immunoblot analysis of whole-cell lysates from matched androgen-dependent (AD) and androgen-independent (AI) sublines of the LAPC-4 human prostate cancer xenograft, for expression of the 185-kDa HER-2/neu protein (***a***; α-Her2) or the 110-kDa androgen receptor protein (***b***; α-AR). This short exposure demonstrates the difference in HER-2/neu expression between AD and AI samples; longer exposure confirmed expression of low levels of HER-2/neu protein in all androgen-dependent LAPC-4 sublines (not shown). Equal loading and transfer of protein to the immunoblot filter was confirmed by Ponceau S staining for total protein (not shown).

Increased HER-2/neu in androgen-independent xenografts

We determined whether HER-2/neu was differentially expressed in androgen-dependent and androgen-independent sublines of prostate cancer xenografts. We have derived several androgen-independent sublines from the original androgen-dependent LAPC-4 xenograft[25] by castrating male SCID mice with androgen-dependent tumors, waiting for regrowth of androgen-independent tumors, then serially passaging androgen-independent xenografts. The level of HER-2/neu protein expression was increased from 2-fold to 25-fold, with a trend towards enhanced HER-2/neu expression with serial passaging (Fig. 1*a*). We found reduced but detectable levels of AR protein in all androgen-independent LAPC-4 sublines that overexpress HER-2/neu (Fig. 1*b*), analogous to the observation that breast cancers overexpressing HER-2/neu have reduced levels of ER protein[12,13]. Androgen independence was not caused by mutations in AR, as no mutations were found in the sequences of the ligand-binding domain of AR from androgen-independent LAPC4 tumors (data not shown). Therefore, androgen-independent growth is associated with increased levels of HER-2/neu and reduced levels of AR in the LAPC-4 model.

HER-2/neu causes androgen-independent growth

If HER-2/neu affected the AR signaling pathway, then overexpression should promote androgen-independent growth *in vitro* and *in vivo*. To test this, we measured the effects of HER-2/neu overexpression in the androgen-dependent prostate cancer cell line LNCaP. We found that the growth rate of LNCaP cells in culture was reduced by more than 50% after 48 hours in androgen-depleted serum, as expected[26]. Addition of dihydrotestosterone restored growth to levels seen with complete media (Fig. 2*b*). Whereas LNCaP cells transformed with the neo vector alone showed a 42% decrease in growth in androgen-deprived medium, two independent subclones of LNCaP cells infected with a retrovirus overexpressing HER-2/neu protein (LH2-K, LH2-N) (Fig. 2*a*) showed only a 15% decrease (Fig. 2*c*). Thus, HER-2/neu can partially rescue LNCaP cells from growth arrest induced by androgen deprivation *in vitro*.

We measured the effects of HER-2/neu on the *in vivo* growth of LNCaP cells in castrated male mice, in which the residual androgen level is insufficient to maintain growth of androgen-dependent prostate cancer cells[25,27,28]. Intact or castrated male SCID mice injected subcutaneously with LNCaP/Neo or LNCaP/HER-2 cells were examined weekly for evidence of tumor formation and scored as positive when tumors greater than 0.5 cm in diameter were detected. HER-2/neu conferred a modest growth advantage in intact male mice (Fig. 3*a*), but shortened the latency for tumor formation by 50% (from 30 to 15 weeks) in castrated males (Fig. 3*b*). Tumors expressing HER-2/neu were also larger and produced higher levels of circulating PSA in the serum (data not shown). Thus, HER-2/neu can substitute for androgen to cause prostate cancer cells to grow *in vivo*.

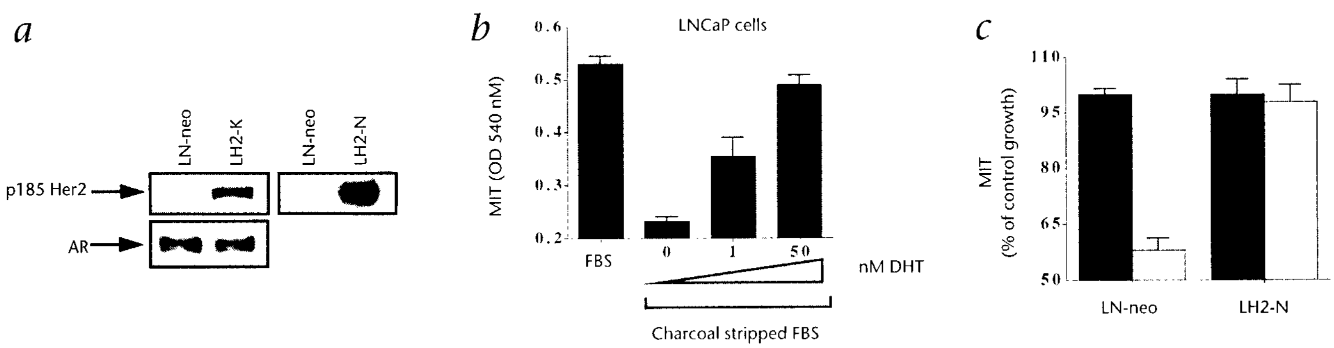
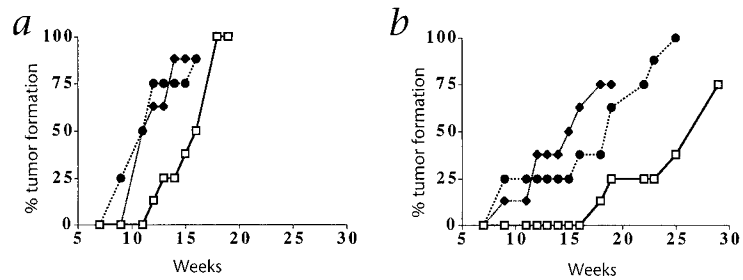


Fig. 2 Effect of HER-2/neu on growth of androgen-dependent prostate cancer cells *in vitro* in the absence of androgen. Androgen-dependent LNCaP prostate cancer cells were infected with retrovirus expressing the control Neo gene or HER-2/neu, and stable lines (LN-Neo and LH2-N, respectively) were derived by selection in G418. ***a***, Immunoblot analysis of HER-2/neu (p185Her2) and androgen receptor (AR) expression. Endogenous levels of HER-2/neu protein expression were observed in LN-Neo cells with longer exposure (not shown). ***b***, The effect of androgen on growth of parental LNCaP cells. Cells were plated in media supplemented with either 10% fetal bovine serum (FBS) or 10% charcoal-stripped FBS with 0 nM, 1 nM or 50 nM dihydrotestosterone (DHT), and MTT assays were done after 48 h. Data represent mean absorbance readings at 540 nM ±s.d. from triplicate wells. ***c***, LN-Neo and LH2-N cells were plated in media supplemented with either 10% FBS (■) or 10% charcoal-stripped FBS (□), and MTT assays were done after 48 h. MTT values for charcoal-stripped FBS are expressed as % relative to complete FBS (representative experiment from seven independent experiments).

Fig. 3 Effect of HER-2/neu overexpression on tumorigenicity of prostate cancer cells in intact and castrated male SCID mice. Parental LNCaP cells (□) or sublines that overexpress HER-2/neu (LH2-N, ◆; and LH2-K, ●) were injected into intact (***a***) and castrated (***b***) male mice (n = 8 for each condition). Tumor size was measured weekly by calipers, and tumors were scored as positive when greater than 0.5 cm in any dimension. Data are presented as % of animals that developed a tumor versus time. Serum PSA levels were comparable (data not shown).



Increased PSA levels in HER-2/neu-expressing prostate cells

The ability of HER-2/neu overexpression to cause androgen-independent growth in prostate cells might be explained by activation of the AR signaling pathway in a ligand-independent fashion. If the model is correct, there should be evidence of AR activation in cells expressing HER-2/neu in the presence of low concentrations or the complete absence of androgen. We assessed the effects of HER-2/neu on the expression of PSA, a well-characterized prostate-specific gene whose transcription is strictly regulated by androgen[29]. Immunoblot analysis demonstrated an increase in endogenous PSA protein in lysates from LNCaP cells overexpressing HER-2/neu (Fig. 4*a*). LNCaP/HER-2 cells also secreted sixfold to sevenfold more PSA than did LNCAP/Neo cells, and this level was enhanced by the addition of R1881 (Fig. 4*b*). These findings demonstrate that activation of the AR-responsive PSA gene mediated by HER-2/neu does not require exogenously added androgen, and that HER-2/neu augments PSA secretion in response to androgen, indicating that there may be cross-talk between the HER-2/neu and AR pathways.

HER-2/neu enhances PSA transcription

The PSA promoter/enhancer contains high-affinity AR binding sites and functions in an androgen-dependent manner[30,31]. To measure the effects of HER-2/neu on this response, we co-transfected LNCaP cells with the PSA-P/E-luc reporter[32] and HER-2/neu or the empty vector, then cultured the cells in phenol red-free media in the absence of serum to allow precise control of androgen concentration. In four independent experiments, HER-2/neu activated the PSA promoter/enhancer construct sixfold to sevenfold in the absence of added androgen (Fig. 5*a*). LNCaP cells contain a mutation in the AR hormone binding domain that can alter AR function. To eliminate the possibility of any effect of this mutation on the HER-2/neu response, we used the androgen-dependent prostate cancer cell line LAPC-4, which contains no mutations in exons 2–8 of AR (ref. 25). HER-2/neu activated the PSA promoter/enhancer 15-fold in LAPC-4 cells in the absence of androgen (Fig. 5*a*). Next, we assessed the effect of HER-2/neu in combination with androgen. R1881 activated PSA-P/E-luc 3-fold to 50-fold in LNCaP/Neo cells at concentrations of 0.03, 0.1 and 1.0 nM (Fig. 5*b*). In the absence of R1881, HER-2/neu activated the PSA-P/E-luc reporter sevenfold, and this response was enhanced at all doses of androgen tested (Fig. 5*b*), indicating that HER-2/neu activates the PSA promoter/enhancer in the absence of androgen but does not prevent further responsiveness to androgen.

HER-2/neu-mediated PSA activation requires androgen receptor

To assess the role of AR in this HER-2/neu-mediated signal, we determined whether the anti-androgen drug casodex, which functions as a competitive inhibitor for androgen binding to AR, could block the HER-2/neu effect. Casodex inhibited PSA-P/E-luc activation by R1881 in LNCaP/Neo cells (Fig. 5*b*) but had no effect on ligand-independent PSA-P/E-luc activation by HER-2/neu. The results are consistent with an AR-independent effect of HER-2/neu or an AR-dependent effect that does not require ligand–receptor interaction. To distinguish between these, we assessed the effects of HER-2/neu on PSA-P/E-luc in the hamster kidney epithelial line TS-13 (ref. 33) that, unlike LNCaP cells, does not have a highly active AR pathway. PSA-P/E-luc alone did not function in these cells unless the AR pathway was reconstituted by the transfection of AR and the addition of R1881 (Fig. 6*a*). HER-2/neu activated PSA-P/E-luc 19-fold in TS-13 cells in the absence of added ligand when the cells were co-transfected with AR. The combination of HER-2/neu, AR and R1881 elicited a 71-fold increase in PSA-P/E-luc activity compared with a 10-fold increase with AR and R1881. These results establish that ligand-independent induction of PSA transcription by HER-2/neu requires a functional AR pathway, and that HER-2/neu and androgen act synergistically.

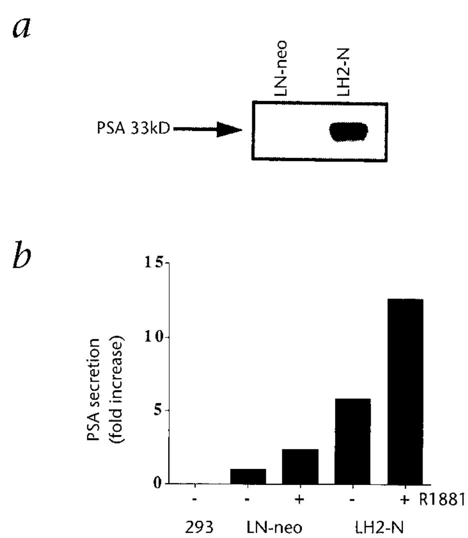


Fig. 4 Effect of HER-2/neu on levels of the androgen-regulated PSA protein. ***a***, Immunoblot analysis of whole-cell lysates from LNCaP cells expressing the control Neo gene (LN-Neo) or HER-2/neu (LH2-N), for expression of PSA protein. This short exposure demonstrates the effect of HER-2/neu expression on PSA protein levels; longer exposure confirmed expression of PSA protein in LN-Neo cells (not shown). Equal loading and transfer of protein to the immunoblot filter was confirmed by Ponceau S staining for total protein (not shown). ***b***, PSA protein concentration in supernatant was measured by ELISA after 24 h of exposure to serum-free media with (+) or without (–) 1 nM R1881. Data are expressed as fold increase relative to LN-Neo cells in the absence of R1881; the actual concentration of PSA protein in LN-Neo cells without R1881 was 0.78 ng/ml. The human kidney epithelial cell line 293 served as a negative control and failed to secrete any detectable PSA protein.

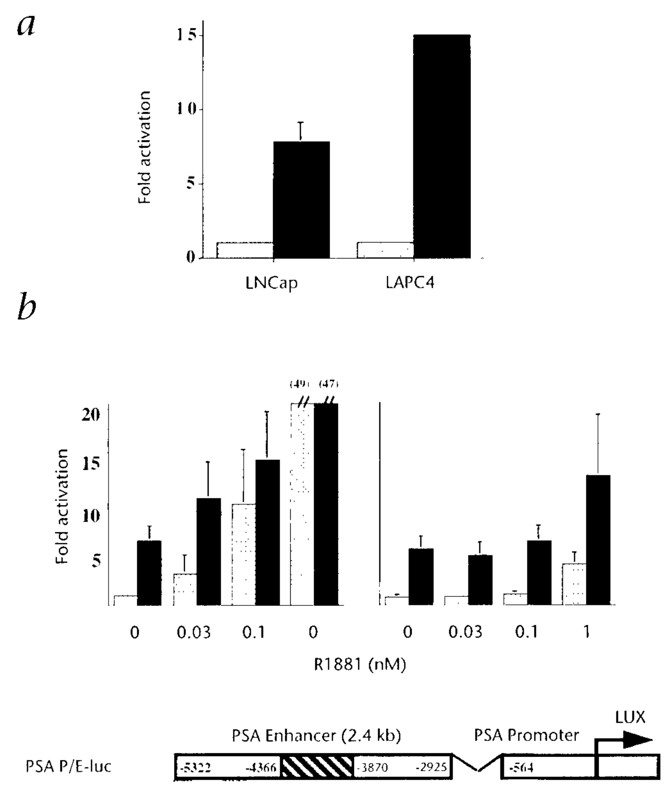


Fig. 5 Effects of HER-2/neu on androgen-dependent PSA transcription. ***a***, Androgen-dependent LNCaP or LAPC-4 cells transfected with the PSA P/E-luc reporter plasmid (bottom) in conjunction with a Neo (□) or HER-2/neu (■) expression vector were plated in serum-free, phenol red-free media. A plasmid expressing GFP was included as a transfection control. Luciferase activity was measured after 48 h. Data (a representative experiment from a total of four) are expressed as fold activation relative to the luciferase activity in LNCaP or LAPC-4 cells transfected with Neo, which is designated as onefold. The transfection efficiency was similar for Neo- and HER-2/neu-transfected cells, as measured by the percentage of flourescent green cells expressing GFP (not shown). ***b***, LNCaP/neo cells (□) or LNCaP/Her-2 cells (■) were transfected with PSA P/E-luc and cultured in serum-free media supplemented with increasing doses of R1881 (left; $n = 4$ for each) or R1881 and 5.0 uM casodex (right; $n = 3$ for each). Luciferase activity was measured after 48 h. Data are expressed as fold activation relative to LNCaP/Neo cells in the absence of R1881 or casodex, which is designated as onefold. Luciferase activation with 1.0 nM R1881 was 49-fold in LNCaP/Neo cells and 47-fold in LNCaP/Her-2 cells.

HER-2/neu 'superactivates' the AR pathway activation

The PSA-P/E-luc construct[32] encodes 2,900 bp of sequence containing one well-defined high-affinity AR binding site in the promoter[34] and a 496-bp enhancer[30]. The enhancer encodes a second high-affinity AR binding site and at least five other non-consensus AR binding sites, as defined by DNAse I footprinting studies (Y.S. and M.C., unpublished data). We localized the effect of HER-2/neu on PSA transcription to specific regions of the promoter/enhancer by constructing two artificial reporters, one containing the 496-bp enhancer (PSA-E E4-CAT) and a second containing the high-affinity AR binding site from the PSA promoter (ARE-I E4-CAT). Both PSA-E E4-CAT and ARE-I E4-CAT were activated in TS-13 cells by R1881 and co-transfection of AR (Fig. 6*b–d*). In the presence of AR, HER-2/neu 'superactivated' PSA-E E4-CAT at three different doses of R1881, as much as 30-fold above the level seen with AR and R1881 alone (Fig. 6*b* and *c*). Using the ARE-I E4-CAT reporter, transfection of HER-2/neu had no effect beyond that induced by the combination of R1881 and AR, even at low doses of R1881 and higher doses of HER-2/neu plasmid (Fig. 6*d*). Similar results were obtained in LNCaP cells expressing endogenous AR (data not shown). Thus, the synergistic interaction between HER-2/neu and the AR pathway can be localized to a 496-bp region of the PSA enhancer, but cannot be recapitulated using a single high-affinity AR binding site reporter.

Discussion

A principal clinical problem in prostate cancer is the conversion of androgen-sensitive tumors to a hormone-refractory state after treatment with anti-androgen therapy. The molecular basis for androgen independence is unknown. Here we show that overexpression of the HER-2/neu receptor tyrosine kinase may be one mechanism. Increased endogenous HER-2/neu expression is associated with androgen independence in the LAPC-4 xenograft model, and forced overexpression of HER-2/neu converts androgen-dependent LNCaP prostate cancer cells to androgen-independence. HER-2/neu exerts this effect through modulation of the AR signal transduction pathway. Specifically, HER-2/neu activates transcription of PSA, an androgen-dependent serum marker of disease progression that usually correlates with tumor burden in patients. HER-2/neu and androgen also function synergistically to 'superactivate' PSA transcription, particularly at low androgen concentrations.

Previous studies of HER-2/neu expression in prostate cancer report conflicting results. Most groups have focused on radical prostectomy samples, which rarely contain androgen-independent disease, and report frequencies of HER-2/neu overexpression that vary widely[17,18,20–23]. Less is known about the frequency of HER-2/neu expression in androgen-independent prostate cancer, mostly because these tumors are rarely biopsied. However, patients with end-stage, hormone-resistant disease have elevated serum levels of the Her2/neu extracellular domain[24]. Future studies using well-defined HER-2/neu detection reagents are needed to clarify this controversy.

It will also be important to determine if other kinases can activate the AR pathway and confer androgen-independent growth. The IGF-1 receptor is of particular interest, as serum IGF-1 levels predict risk of prostate cancer[45] and inhibition of the receptor impairs prostate cancer cell growth[46]. IGF-1, KGF and EGF activate the AR pathway in the absence of ligand[10], indicating that the effects of HER-2/neu may not be unique. The fact that the androgen antagonist casodex can block the effects of IGF-1, KGF and EGF but not HER-2/neu on AR function may indicate important differences. The failure of casodex to block PSA induction by HER-2/neu is consistent with clinical androgen-independent prostate cancer, which arises in patients treated with anti-androgens, and indicates that HER-2/neu acts on the AR pathway distal to the interaction between ligand and receptor.

The biochemical details of the cross-talk between the HER-2/neu and AR pathways are unclear. The fact that HER-2/neu fails to activate a single high affinity AR binding site supports the idea of the involvement of an accessory protein, whose function in an AR-dependent transcription complex might not be measurable on an AR binding site removed from its natural context. Alternatively, the main effect of HER-2/neu may be to optimize AR function on non-optimal AR binding sites rather than to enhance AR function on high-affinity binding sites. HER-2/neu activates the Ras and mitogen-activated protein kinase signaling pathways[37], which may be involved in post-

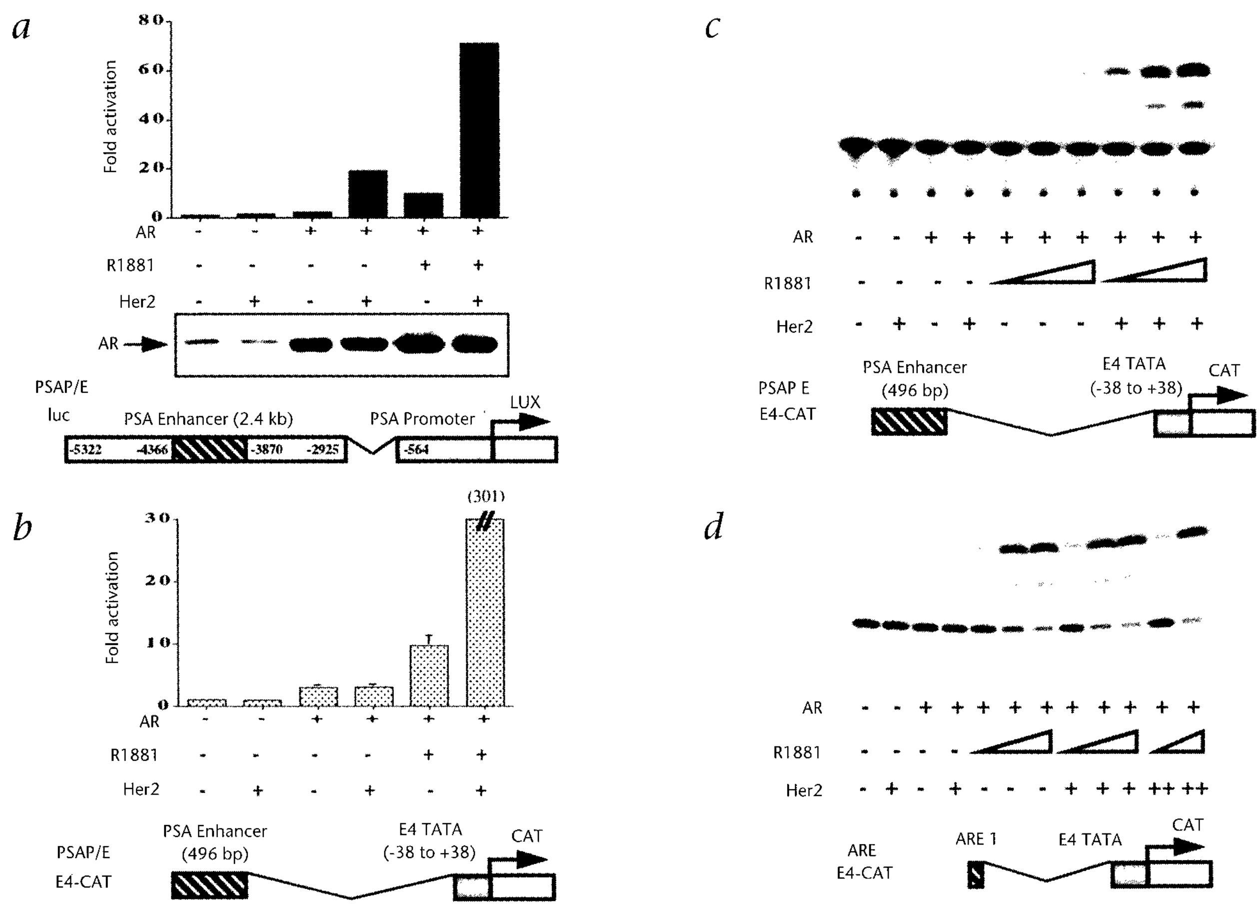


Fig. 6 Effects of HER-2/neu on androgen receptor function. ***a***, TS-13 hamster kidney epithelial cells were transfected with PSA P/E-luc in conjunction with plasmids expressing androgen receptor (AR) and/or HER-2 in various combinations and in the presence or absence of 1.0 nM R1881 (+ or –). Expression of AR was measured by immunoblot analysis of whole-cell lysates. Luciferase results are expressed as fold activation relative to TS13 cells transfected with PSA P/E luc in the absence of AR, HER-2/neu and R1881, which was designated as onefold. ***b***, Similarly, TS-13 cells were transfected with the PSA E E4-CAT reporter construct in the presence or absence of AR, HER-2/neu and/or 1.0 nM of R1881. CAT results were analyzed by thin layer chromatography and quantitated using a phosphorimager. Data are expressed as fold activation relative to TS13 cells transfected with PSA E E4-CAT in the absence of AR, HER-2/neu and R1881, which was designated as onefold, and are the mean (s.d. from three independent experiments. ***c***, Dose–response of the PSA E E4-CAT reporter to R1881, generated by transfection of TS13 cells with AR (HER-2/neu with R1881 at concentrations of 0.1 nM, 0.3 nM and 1.0 nM (wedges). CAT activity was measured by thin layer chromatography. At higher doses of R1881, the PSA E E4-CAT construct was maximally activated and no additional effect of HER-2/neu was observed (data not shown). ***d***, Dose–response of a reporter containing a single androgen response element (ARE E4-CAT) to R1881 with AR (HER-2/neu with R1881 at concentrations of 0.01 nM, 0.1 nM and 1.0 nM (wedges). CAT activity was measured by TLC. The two right lanes contain higher doses of HER-2/neu plasmid (++).

translational modification of ER (refs. 9,38). Given the results of studies of nuclear receptors for thyroid hormone, retinoic acid and others, it is likely that a combination of post-translational modifications as well as alterations in the assembly of multicomponent transcription complexes may occur[39,40].

Recognizing that there is cross-talk between tyrosine kinase receptor signaling and the AR pathway has clinical implications. Strategies to inhibit the relevant tyrosine kinase receptor would be expected to convert androgen-independent prostate cancer back to a hormone-sensitive state. For HER-2/neu, this might be tested using a recently developed monoclonal antibody that blocks HER-2/neu function and has clinical efficacy in breast cancer when combined with chemotherapy[41,42]. Alternatively, a detailed understanding of the biochemical effects of receptor tyrosine kinase signaling on AR function might provide new drug development insights into targeting the AR pathway downstream of the point of ligand–receptor interaction.

Methods

Cell lines and xenografts. Androgen-dependent and androgen-independent sublines of the LAPC-4 xenograft were derived as described[25]. LNCaP/HER2 and LNCaP/Neo cells were derived by infection with the pLNSXHer2 or pLNSXNeo retrovirus, respectively[43], and selection in 500 ug/ml G418. Tumorigenicity was measured by the injection of 1×10^5 cells suspended in 100 ul of Matrigel (Collaborative Biomedical, Bedford, Massachusetts) subcutaneously into the flanks of intact or castrated male SCID mice. Tumor size was measured weekly in three dimensions using calipers as described[25]. For MTT assays, 1.5×10^5 LNCaP cells were seeded into 24-well plates in phenol red-free RPMI supplemented with 10% fetal bovine serum (FBS) overnight. After 12 h, cells were washed, then re-supplied with phenol red-free RPMI media with complete 10% FBS or 10% FBS that had been treated with charcoal dextran to remove steroid hormone (Omega Scientific, Tarzana, California). Dihydrotestosterone (Sigma) was added at defined concentrations. After 48 h, MTT assays were done in triplicate: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (Sigma) was added to a final concentration of 5 mg/ml for 4 hours at 37 °C on a multiwell spectrophotometer, and absorbance was measured at 540 nm.

Protein expression assays. HER-2/neu and PSA expression were measured by immunoblot using antibody against c-Neu (Calbiochem, La Jolla, California) at a dilution of 1:1,000 or antibody against PSA (Dako, Carpinteria, California) at a dilution of 1:250. AR was immunoprecipitated from cell extracts using anti-AR antibody (PharMingen, San Diego, California) at a dilution of 1:1,000, followed by immunoblot analysis. PSA secretion was quantitated by ELISA (Hybritech, San Diego, California) of tissue culture supernatant. Equal cell numbers were plated in phenol red free RPMI containing 10% FBS. Cells were allowed to attach for 24 h, then the media was changed to serum-free RPMI with or without 1 nm of the synthetic androgen R1881 (NEN). After another 24 h, 50 µl of supernatant was assayed for PSA by ELISA.

Transfections. PSA-P/E-luc (ref. 32) was provided by A. Belldegrun (University of California at Los Angeles). PSA-E E4-CAT was constructed by subcloning the 496-bp enhancer fragment into E4-CAT (ref. 44). ARE-I E4-CAT was constructed by subcloning a double-stranded oligonucleotide encoding the ARE-I site from the PSA promoter (AGAACAGCAAGTGCT)(ref. 34) into E4-CAT. LNCaP or LAPC-4 cells were maintained in phenol red-free RPMI supplemented with 10% FBS. Cells (2×10^5) were plated in 6-well plates overnight, then transfected using Tfx-50 (Promega) in 2 ml serum-free OptiMEM (Life Technologies). TS13 cells were transfected using calcium phosphate. After 1 h, 2 ml of serum-free media was added, containing varying amounts of the testosterone analog R1881 without or with 5 uM casodex (Zeneca, Dallas, Texas). After 48 h, cells were collected in luciferase assay lysis buffer and analyzed as described (Promega) and normalized to protein content. CAT assays were done as described[45].

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Mitogen-Activated Protein Kinase Kinase Kinase 1 Activates Androgen Receptor-Dependent Transcription and Apoptosis in Prostate Cancer

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Mitogen-activated protein (MAP) kinases phosphorylate the estrogen receptor and activate transcription from estrogen receptor-regulated genes. Here we examine potential interactions between the MAP kinase cascade and androgen receptor-mediated gene regulation. Specifically, we have studied the biological effects of mitogen-activated protein kinase kinase kinase 1 (MEKK1) expression in prostate cancer cells. Our findings demonstrate that expression of constitutively active MEKK1 induces apoptosis in androgen receptor-positive but not in androgen receptor-negative prostate cancer cells. Reconstitution of the androgen receptor signaling pathway in androgen receptor-negative prostate cancer cells restores MEKK1-induced apoptosis. MEKK1 also stimulates the transcriptional activity of the androgen receptor in the presence or absence of ligand, whereas a dominant negative mutant of MEKK1 impairs activation of the androgen receptor by androgen. These studies demonstrate an unanticipated link between MEKK1 and hormone receptor signaling and have implications for the molecular basis of hormone-independent prostate cancer growth.

Steroid hormones play a critical role in the development and maintenance of multiple organs, including mammary glands (estrogens), the uterine lining (progesterone), and the adrenal medulla (glucocorticoids) (19, 26). In addition to responding to their ligands, steroid hormone receptors are modified by kinase signaling pathways which directly or indirectly alter the biological response to hormones (27). In the case of the androgen receptor, one model system for functional studies is the prostate gland. Prostate development is dependent on androgen, and normal prostate secretory epithelial cells undergo apoptosis in response to androgen withdrawal (9). Prostate cancer cells are also dependent on androgen for growth but eventually acquire the ability to proliferate in the absence of androgen in patients after prolonged anti-androgen drug therapy (2, 35). Although androgen independent, these cells continue to express androgen-responsive genes, indicating ligand-independent activation of the androgen receptor signaling pathway. Defining the mechanism for this conversion to androgen independence will have important implications in prostate cancer therapy (47).

A number of protein kinase signaling pathways have been implicated in androgen receptor signaling. Protein kinase A can activate androgen receptor-mediated gene transcription in the absence of androgen (23, 38). The protein kinase C activator and tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate negatively regulates androgen receptor-mediated gene transcription through a presumed interaction of c-jun and androgen receptor (43). Epidermal growth factor (EGF), keratinocyte growth factor (KGF), or insulin-like growth factor 1 (IGF-1) can activate transcription from androgen receptor-regulated genes in prostate cancer cells (11, 42). Transgenic mice expressing KGF under the control of the hormone-responsive mouse mammary tumor virus promoter develop prostatic hyperplasia, suggesting that tonic exposure to certain growth factors results in dysregulated prostate growth in vivo (28). These reports establish that interactions between androgen receptor and non-steroid receptor signaling pathways exist, but the molecular details are unclear.

Because many of these growth factors activate the mitogen-activated protein (MAP) kinase pathway, we hypothesized that isolated activation of this pathway may affect androgen receptor-mediated gene regulation and the prostate cancer cell phenotype. In particular, we examined the effect of MAP kinase kinase kinase 1 (MEKK1) signaling in prostate cancer cells. Activation of MEKK1 results in the downstream activation of MKK4 (SEK1) and subsequently JNK (36), as well as phosphorylation of IκB kinase leading to the release of NF-κB (30, 37, 56). JNK activation is associated with diverse outcomes which vary in different cell types and in the presence of concurrent signals from other pathways. JNK activation is necessary for cellular transformation by the Bcr-Abl oncogene (14, 41) but is also associated with apoptosis in response to growth factor deprivation or withdrawal of extracellular matrix (anoikis) (5, 53). Constitutively active alleles of MEKK1 induce apoptosis in diverse cell types (25, 51). A model for MEKK1-mediated apoptosis has emerged in which genotoxic stress leads to phosphorylation and activation of MEKK1 followed by MEKK1-initiated cleavage of DEVD-directed caspases. MEKK1 is itself a target for cleavage by caspases, which leads to further activation of MEKK1 by removal of a negative regulatory domain (5, 50). Thus, MEKK1 participates in a caspase activation loop which requires both the kinase activity of MEKK1 as well as the caspase recognition site, permitting its cleavage by caspases.

Here we address the role of the MEKK pathway in prostate cancer. Our findings demonstrate that expression of constitutively active MEKK1 leads to apoptosis of androgen receptor-positive but not of androgen receptor-negative prostate cancer

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cells. Reconstitution of the androgen receptor pathway sensitizes prostate cancer cells to MEKK1-induced apoptosis. MEKK1 also activates androgen-regulated gene expression in an androgen receptor-dependent fashion. These data demonstrate cross-talk between the androgen receptor signaling pathway and MEKK1 that results in transcriptional regulation of androgen receptor-regulated genes and apoptosis.

MATERIALS AND METHODS

Cell culture and reagents. LNCaP, PC3, and DU145 human prostate cells were obtained from the American Type Culture Collection and maintained in phenol red-free RPMI with 10% fetal calf serum (FCS) or 10% charcoal-stripped FCS (Gemini, Thousand Oaks, Calif.). LAPC4 cells were derived from a human prostate cancer xenograft implanted in SCID mice and express wild-type androgen receptor (exons 2 to 8) (29). LAPC4 cells were grown in Iscove's medium with 10% FCS. R1881 was used as a synthetic androgen (DuPont-NEN), and Casodex was used as an androgen receptor antagonist (ICI, Cheshire, United Kingdom).

Plasmids, transfections, and retroviral infections. The cDNAs for MEKKΔ (MEKK-dominant active or MEKKΔ-DA) and MEKKΔ(K432M) (MEKK-dominant negative or MEKKΔ-DN) (a kind gift of Michael Karin) were subcloned into pCDNA3 and the retroviral pSRαMSV-tkNeo vector (36). MEKKΔ is a truncated form of MEKK1 in which amino acids 1 to 351 have been deleted and MEKKΔ(K432M) contains a mutation in the ATP-binding site rendering it catalytically inactive. Cells were infected with amphotropically packaged retrovirus and selected in G418. Transient transfection of cells was performed by lipid-mediated gene transfer with Lipofectamine (Gibco-BRL) or TFX-50 (Promega, Madison, Wis.). Successful gene transfer was confirmed by cotransfection with a vector encoding enhanced green fluorescent protein (GFP; Clontech). 2X-TRE-luciferase was used to measure activator protein 1 activity. For androgen receptor-regulated gene transcription, a 600-bp fragment of the prostate-specific antigen (PSA) promoter with an additional 2.4-kb enhancer sequence cloned upstream of luciferase (PSA P/E-luc) was used (39). Additionally, an androgen-regulated reporter vector was created by multimerizing four consensus androgen receptor response elements from the PSA promoter (ARE-I) cloned upstream of the chloramphenicol acetyltransferase (CAT) gene in the pBXG0 vector and referred to as 4X-ARE/E4-CAT (a gift from Michael Carey). For the ZEBRA reporter experiments, pZRE-5/E4-CAT was used with pZEBRA driven by a simian virus 40 enhancer (31). Full-length wild-type androgen receptor was expressed by using a cytomegalovirus-driven plasmid expression vector (a gift of Marco Marcelli) (34). The plasmid pCDNA3-JBD was used to inhibit JNK1 activity. This construct contains the domain of JIP-1 that binds JNK-1 (JBD) cloned into pCDNA3 (14).

The protocol used for transfection of cell lines was as follows. Cells were plated at a density of 5×10^5 cells in a 60-mm-diameter dish on the day prior to transfection. In all cases, the total amount of transfected DNA was kept constant with control vector. For LNCaP and LAPC4 cells, TFX-50 (Promega) was used to transfect cells. A total of 4.4 μg of DNA and 20 μl of lipid reagent was added to the cells in Optimem (Gibco). After a 1-h incubation, medium containing 10% charcoal-stripped serum was added to the cells. For DU145, PC3 and 293T cells, Lipofectamine (Gibco) was used to transfect cells. A total of 4.4 μg of DNA and 18 μl of lipofectamine was added to the cells in Optimem. After a 5-h incubation, medium containing 10% charcoal-stripped serum was added to the cells. In some cases, androgen (R1881) or Casodex was added with the medium containing 10% charcoal-stripped serum. Luciferase assays, CAT assays, and apoptosis measurements were performed 48 h after transfection unless otherwise stated.

Reporter assays. Luciferase activity was measured with a Luciferase Assay Kit (Promega). Cells were lysed in 100 μl of 1× lysis buffer, and 20 μl was used to react with luciferase substrate. Light units were measured with a luminometer. CAT activity was measured with a CAT enzyme-linked immunosorbent assay (ELISA) kit (Boehringer-Mannheim) or by conventional CAT assay as previously described (41). Samples were analyzed by thin-layer chromatography and exposed to a Storm phosphorimager screen. Radioactivity was quantitated by using ImageQuant software.

Kinase assays and Western blots. JNK, ERK, and p38 kinase activity were measured as previously described (41). Briefly, equal numbers of cells were lysed in radioimmunoprecipitation assay buffer and JNK1 (sc474; Santa Cruz), ERK1/2 (Zymed), or p38 (sc535-G; Santa Cruz) was immunoprecipitated with antibodies as indicated. Immunoprecipitates were reacted with the substrates glutathione *S*-transferase (GST)-c-jun (1-79), myelin basic protein, or GST-ATF-2, respectively, in the presence of [γ-^{32}P]ATP and analyzed by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE). In indicated cases, FLAG-tagged JNK1 was immunoprecipitated with anti-FLAG-conjugated beads (Sigma) and reacted with GST-jun as described above. For MEKK Western blots, anti-MEKK1 was used at 0.5 μg/ml (sc252; Santa Cruz) with anti-rabbit horseradish peroxidase secondary (Jackson Laboratories). For androgen receptor Western blots, whole-cell lysates were analyzed by 8% PAGE and reacted with rabbit anti-human androgen receptor antibody (N-20, sc816; Santa Cruz) used at a 1:500 dilution.

Apoptosis assays. Apoptosis was detected morphologically by using acridine orange or transfected GFP. A fluorescent microscope was used to count 200 fluorescent cells per condition, and the percentage of blebbing cells was calculated. Cells were scored by an investigator blinded to the experimental condition. DNA staining of cells was performed with Hoechst 33258. At 48 h after transient transfection, cells were rinsed with phosphate-buffered saline, fixed with paraformaldehyde 4% for 15 min, permeabilized with Triton X-100 0.5%, and then stained in the dark with Hoechst dye at 2.5 μg/ml (53). Chromatin condensation was used as an additional morphologic marker of apoptosis in cells cotransfected with GFP.

Statistical analysis. Statistical analysis was performed by parametric analysis using the paired Student *t* test and Microsoft Excel.

RESULTS

Expression of activated MEKK1 induces apoptosis in androgen receptor-positive but not in androgen receptor-negative prostate cancer cells. The androgen receptor-positive prostate cancer cell line LNCaP is a well-characterized model for the study of androgen receptor-mediated growth and signal transduction (32, 48). We examined the role of the stress-activated MAP kinase signaling pathway in LNCaP cells by utilizing retroviruses expressing a truncated, constitutively active form of MEKK1 (MEKKΔ-DA) and a catalytically inactive mutant containing a point mutation in the ATP binding site (MEKKΔ-DN) (36). At 48 h after infection with retrovirus, MEKKΔ-DA- and MEKKΔ-DN-infected cells expressed similar levels of the truncated MEKK1 protein (Fig. 1a). Biochemical characterization of LNCaP cells stably expressing MEKKΔ-DA demonstrated selective activation of the JNK pathway (sixfold) over parental cells and minimal p38 (twofold) or ERK activation (Fig. 1b). After antibiotic selection, populations of cells stably expressing MEKKΔ-DA were derived. In five independent experiments, these cells consistently demonstrated reduced MEKKΔ-DA protein expression compared with MEKKΔ-DN (Fig. 1a). These data suggest that high-level expression of MEKKΔ-DA is not well tolerated in LNCaP cells, as reported previously in fibroblasts (25, 51). To look directly for effects on growth, the LNCaP sublines were plated at equal densities, and cells were counted after 5 days in culture. In five independent experiments in which mass populations of cells were selected, LNCaP cells expressing MEKKΔ-DA were difficult to expand compared with Neo control cells or MEKKΔ-DN-expressing cells (Fig. 2b). These findings demonstrate that the expression of MEKKΔ-DA impairs the expansion of LNCaP cells in vitro.

To determine whether MEKKΔ-DA functioned similarly in other prostate cancer cell lines, we extended our analysis to DU145 and PC3 cells, which differ from LNCaP because they do not express androgen receptor and do not require androgen for growth. DU145 and PC3 cells were infected with retrovirus expressing MEKKΔ-DA or Neo control, and sublines which expressed MEKKΔ-DA were derived by antibiotic selection (Fig. 2a). Unlike LNCaP cells, there was no difficulty in expanding MEKKΔ-DA-expressing DU145 and PC3 cells. To assess the effect of MEKKΔ-DA on PC3 and DU145 growth, each subline was plated at an equal density, and cell counts were determined after 5 days and compared to Neo control sublines. In contrast to LNCaP cells, MEKKΔ-DA expression did not impair the growth of PC3 or DU145 cells in three experiments with independently selected sublines (Fig. 2c). We then asked whether our difficulty in expanding the LNCaP cells which stably express MEKKΔ-DA was due to cell cycle arrest or an increase in cell death. Cell cycle analysis of propidium iodide-stained MEKKΔ-DA cells showed no differences in the percentage of cells in G_1, S, or G_2 compared with the Neo control (Fig. 2c). However, when the morphology of the cells was examined after staining with acridine orange, we noted

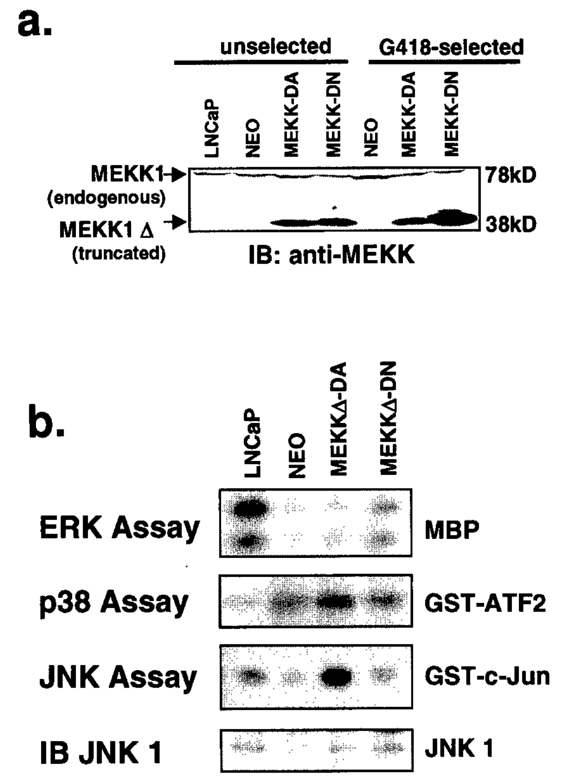

FIG. 1. Biochemical characterization of LNCaP cells stably expressing mutant MEKK1. (a) MEKK1 immunoblot blot of LNCaP cells before and after G418 selection. LNCaP cells were infected with retrovirus pSRαMEKKΔ-DA or pSRαMEKKΔ-DN or Neo control virus. Whole-cell lysates were prepared from cells on the day after retroviral infection prior to G418 selection (unselected) or 2 weeks after G418 selection (G418-selected). The expression of truncated MEKK1 protein is similar in cells infected with pSRαMEKKΔ-DA or pSRαMEKK-DN immediately after infection, suggesting similar viral titers. After G418 selection, however, surviving cells express lower amounts of MEKKΔ-DA. Full-length MEKK1 is a 190-kDa protein not shown on this blot. This C-terminal-directed antibody recognizes a cleaved form of endogenous MEKK1 which runs at approximately 78-kDa and is the same in all lanes (5, 56). Equal protein loading was confirmed by protein assay and Ponceau S staining. (b) In vitro kinase assays of LNCaP sublines stably expressing MEKK isoforms as indicated. Cells expressing MEKKΔ-DA show approximately sixfold activation of JNK activity compared with control cells but only twofold activation of p38 kinase activity. A JNK1 immunoblot demonstrates the relative amounts of immunoprecipitated JNK1 in the different sublines.

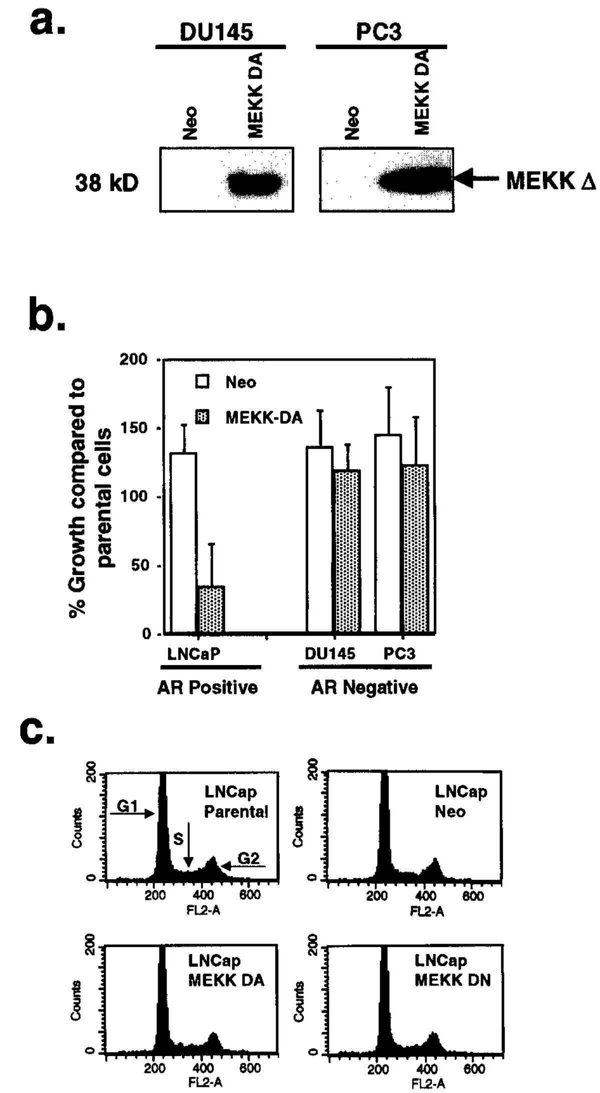

FIG. 2. Effect of stable expression of MEKKΔ-DA in prostate cancer cell lines. (a) MEKK immunoblot of DU145 and PC3 cells after infection with pSRαMEKKΔ-DA retrovirus or Neo control virus. Whole-cell lysates were prepared from cells 2 weeks after G418 selection. Equal protein loading was confirmed by protein assay and Ponceau S staining. (b) Change in cell number of prostate cancer cell lines stably expressing MEKKΔ-DA. After antibiotic selection, DU145, PC3, and LNCaP sublines were plated at 100,000 cells per 60-mm-diameter plate, and the cell numbers were calculated after 5 days in culture. Data are expressed as the percentage of cells on day 5 in the sublines (Neo or MEKKΔ-DA) compared with the parental line. Experiments were performed in duplicate, and this is one representative of three independently derived stable cell lines. (c) Cell cycle analysis of LNCaP cells stably expressing mutant MEKK isoforms. Subconfluent LNCaP cells growing in 10% FCS were permeabilized, stained with propidium iodide, and analyzed on a Becton Dickinson flow cytometer. There are no differences between MEKKΔ-DA-expressing cells and Neo control cells with regard to G_1, S, and G_2 peaks.

changes in LNCaP cells stably expressing MEKKΔ-DA, such as cytoplasmic blebbing and detachment, that are suggestive of apoptosis.

To determine whether MEKKΔ-DA induces apoptosis in LNCaP cells, a quantitative, short-term transient-transfection assay was utilized. LNCaP cells were transiently cotransfected with MEKKΔ-DA and a vector expressing GFP to visualize the morphology of the transfected cells. Approximately 25% of GFP-positive cells cotransfected with MEKKΔ-DA showed cytoplasmic blebbing, a morphologic feature of apoptosis, whereas GFP-positive cells cotransfected with control vector or kinase-inactive MEKKΔ-DN did not (Fig. 3). Our conclusion that MEKK1 induces apoptosis was confirmed independently by the demonstration of chromatin condensation in a high fraction of GFP-positive cells in plates transfected with MEKKΔ-DA but not with the control Neo vector (Fig. 3c). We conclude that the difficulty in expanding LNCaP cells expressing MEKKΔ-DA is most likely a result of the induction of apoptosis, a finding similar to those of earlier studies with fibroblasts and T cells (16, 25).

Next, we analyzed the effect of MEKKΔ-DA expression on apoptosis in androgen receptor-negative DU145 cells and PC3 cells by using the transient-cotransfection assay described above. In contrast to LNCaP cells, there was no increase in the morphologic features of apoptosis in DU145 cells or PC3 cells expressing MEKKΔ-DA at 48 h after transfection (Fig. 3). We extended the analysis in PC3 and DU145 cells to 72 and 96 h after transient transfection with MEKKΔ-DA to look for delayed effects on apoptosis, but we continued to find no differ-

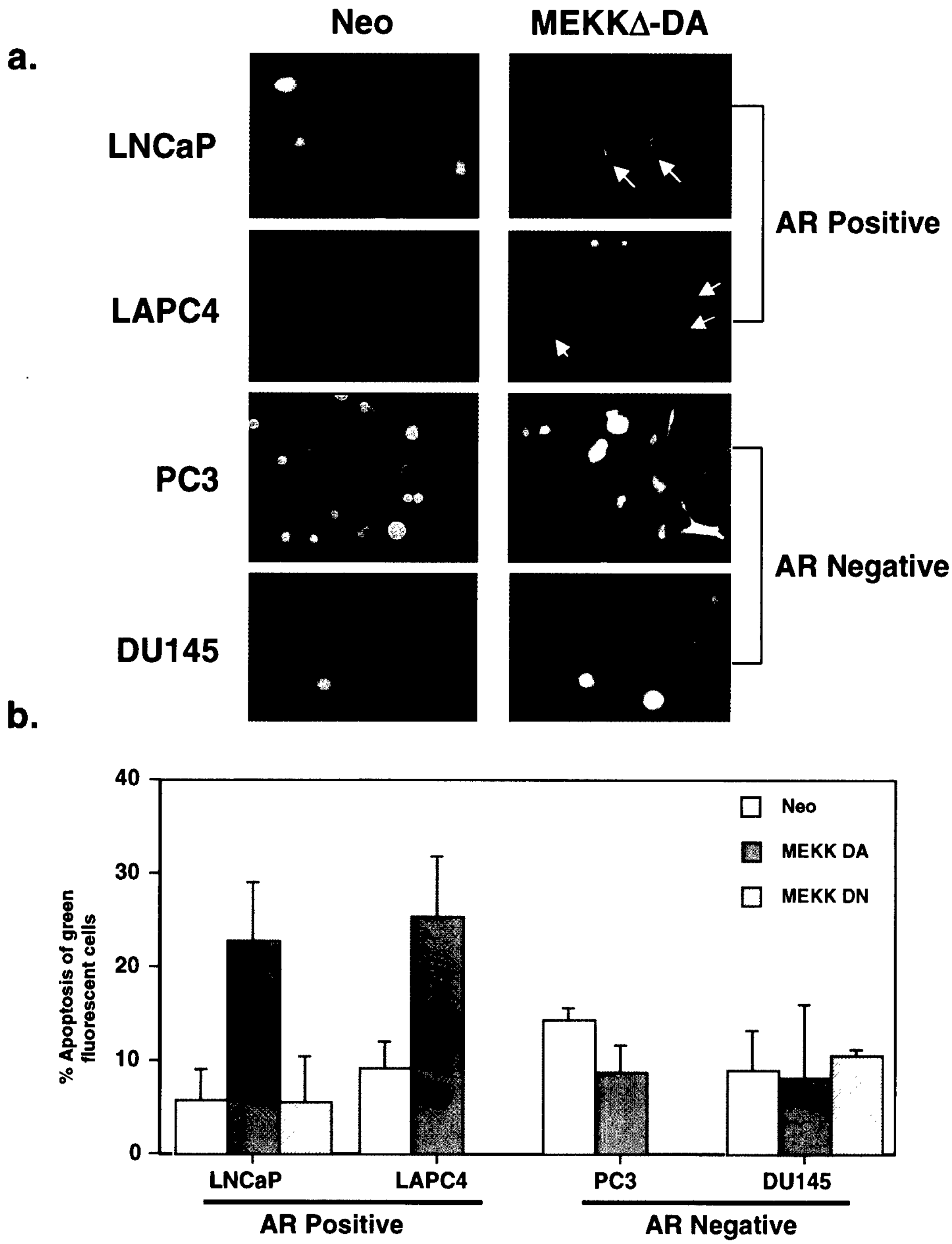

FIG. 3. Effect of transient MEKKΔ-DA expression on apoptosis in androgen receptor-negative and androgen receptor-positive prostate cancer cell lines. (a) LNCaP cells were transiently transfected with GFP (0.4 μg) and cotransfected with Neo or MEKKΔ-DA (0.6 μg), and the total amount of transfected DNA (4 μg) was kept constant with Neo control vector. DU145, PC3, and LAPC4 cells were transfected with 3.6 μg of pCDNA3 Neo or MEKKΔ-DA and cotransfected with GFP (0.4 μg). Apoptotic cells demonstrate cytoplasmic blebbing (arrows). Cells were scored for apoptosis 48 h after transfection. The transfection efficiencies for each cell line are as follows: LNCaP Neo, 40 to 50%; MEKKΔ-DA, 40 to 50%; DU145 Neo, 20 to 30%; MEKKΔ-DA, 20 to 30%; PC3 Neo, 30 to 40%; MEKKΔ-DA, 30 to 40%; LAPC4 Neo, 20 to 30%; MEKKΔ-DA, 20 to 30%. (b) Graph represents three independent experiments in which 200 green fluorescent cells were counted and scored for cytoplasmic blebbing 48 h after transfection. For LNCaP cells, these experiments were also performed with transfected kinase-inactive MEKKΔ-DN (3 μg) which did not induce apoptosis. (c) LNCaP cells were transiently transfected with GFP (0.4 μg) and cotransfected with Neo or MEKKΔ-DA (0.6 μg), and the total amount of transfected DNA (4 μg) was kept constant with Neo control vector. DU145 cells were transfected with 3.6 μg of pCDNA3 Neo or MEKKΔ-DA and cotransfected with GFP (0.4 μg). At 48 h after transfection, cells were stained with the DNA dye Hoechst 33258, and GFP-positive cells were scored for chromatin condensation. There was no increase in chromatin condensation in DU145 cells transfected with Neo or MEKKΔ-DA. White arrows indicate GFP-positive cells, and yellow arrows indicate GFP-positive cells showing chromatin condensation.

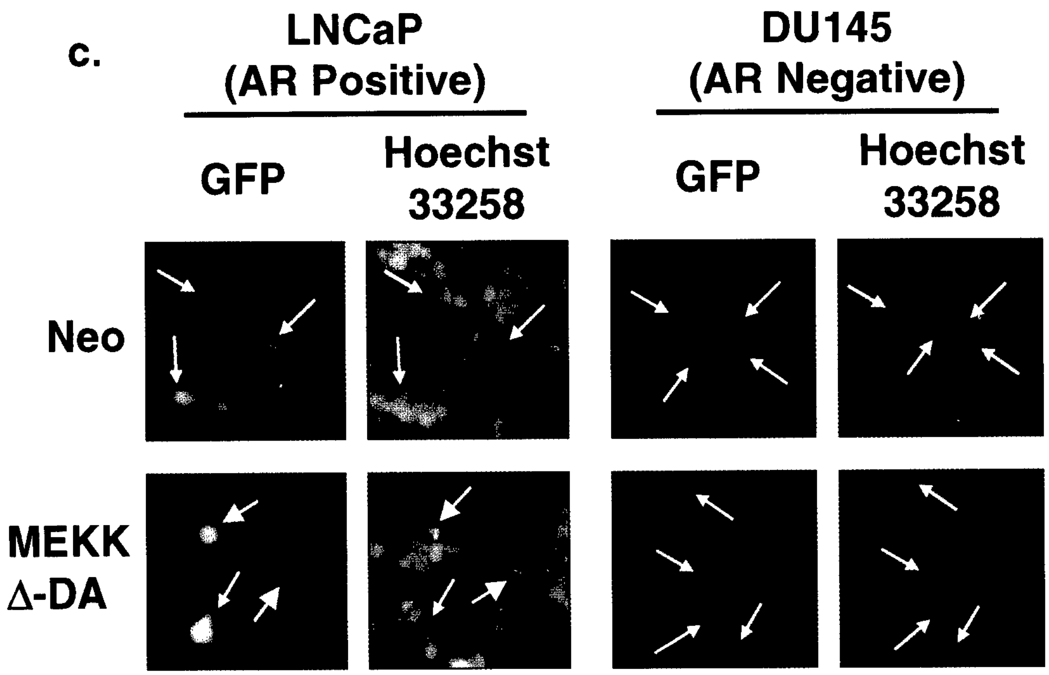

FIG. 3—*Continued.*

ences in apoptosis between Neo- and MEKKΔ-DA-transfected cells (data not shown). These data demonstrate that the ability of MEKKΔ-DA to impair growth or induce apoptosis is restricted to certain prostate cancer cell lines.

Because MEKKΔ-DA-induced apoptosis occurred in the androgen receptor-positive LNCaP cell line but not in two androgen receptor-negative prostate cell lines, we analyzed the effect of MEKKΔ-DA in another model of androgen receptor-positive prostate cancer developed in our laboratory (29). LAPC4 cells express wild-type androgen receptor (exons 2 to 8) and secrete PSA. Similar to LNCaP, transient transfection of MEKKΔ-DA induced apoptosis in LAPC4 cells (Fig. 3). These data suggest that androgen receptor-positive prostate cancer cells are sensitive to MEKKΔ-DA-induced apoptosis, whereas androgen receptor-negative cells are not.

MEKKΔ-DA-induced apoptosis is JNK independent but caspase dependent. One reason for the failure of DU145 and PC3 cells to undergo apoptosis may be a defect in the ability of MEKKΔ-DA to activate the JNK pathway in these cells. To address this question, we tested the ability of MEKKΔ-DA to activate JNK and AP-1 transcriptional activity in androgen receptor-positive and androgen receptor-negative cell lines. To allow for differences in transfection efficiencies between prostate cancer cell lines, we transfected LNCaP, DU145, and PC3 cells with MEKKΔ-DA and FLAG-tagged JNK1 and performed an in vitro kinase assay with anti-FLAG immunoprecipitated JNK1 (Fig. 4a). As expected, an anti-FLAG immunoblot showed different levels of immunoprecipitated FLAG-JNK1 protein from the three cell lines, a finding consistent with distinct transfection efficiencies. However, JNK was activated four- to sixfold by MEKKΔ-DA in all three cell lines when adjusted to the level of immunoprecipitated JNK protein. Cotransfection of the 2X-TRE-luciferase reporter construct revealed similar findings of AP-1 activation in response to the transfection of MEKKΔ-DA in all three cell lines (data not shown). These data demonstrate that MEKKΔ-DA is capable of JNK activation in prostate cancer cell lines regardless of their sensitivity to MEKKΔ-DA-induced apoptosis.

To directly test the role of the JNK pathway in MEKK-mediated apoptosis in androgen receptor-positive cell lines, we examined the effects of JNK inhibition in the transient-transfection assay. LNCaP cells were cotransfected with MEKKΔ-DA and JBD, a truncated form of JIP-1, a selective inhibitor of JNK1 (14). As suspected from related studies in other cell types, JBD inhibited MEKKΔ-DA-mediated activation of jun as measured by a gal4-jun reporter system, thus confirming the activity of JBD in LNCaP cells. However, JBD failed to block MEKKΔ-DA-mediated apoptosis, whereas cotransfection of the baculovirus-derived caspase inhibitor p35 did (57) (Fig. 4b). Taken together, these data indicate that MEKKΔ-DA-induced apoptosis is JNK independent but caspase dependent. This conclusion is in agreement with recent studies of MEKK function in fibroblasts (25, 51).

Modulation of androgen receptor function influences the sensitivity of MEKKΔ-DA-induced apoptosis. A major difference between the prostate cancer cell lines sensitive to MEKK1-induced apoptosis and those resistant to MEKK1-induced apoptosis is the presence of a functional androgen receptor pathway. The LNCaP and LAPC4 prostate cancer cell lines express the androgen receptor, whereas DU145 and PC3 do not. Based on these observations, we hypothesized that the androgen receptor pathway may be required for MEKK1-induced apoptosis in prostate cancer cells. We used three approaches to test this hypothesis: reconstitution of the androgen receptor pathway in androgen receptor-negative cells, pharmacologic inhibition of the androgen receptor pathway in androgen receptor-positive cells, and amplification of androgen receptor signaling in androgen receptor-positive cells. First, we reconstituted the androgen receptor pathway in DU145 cells by transfecting androgen receptor and treating the cells with androgen. Expression of wild-type androgen receptor with or without androgen did not induce significant levels of apoptosis (Fig. 5a). Expression of MEKKΔ-DA with the androgen receptor in the absence of ligand also did not result in apoptosis. However, the combination of MEKKΔ-DA, androgen receptor, and androgen did induce apoptosis in a dose-response

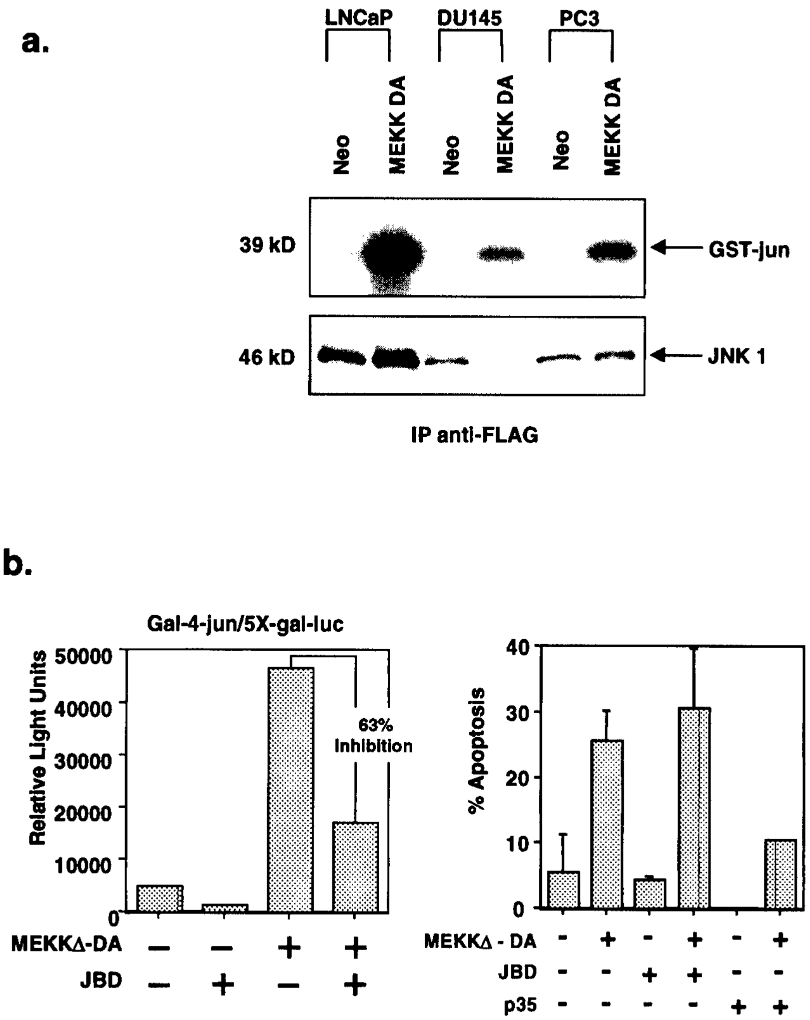

FIG. 4. Role of JNK activation in MEKKΔ-DA-induced apoptosis. (a) Comparison of JNK activation in prostate cancer cell lines in response to MEKKΔ-DA. LNCaP, DU145, and PC3 cells were transfected with FLAG-tagged JNK1 (2 μg) and cotransfected with Neo or MEKKΔ-DA (2 μg). The top panel shows a JNK assay in which 100 μg of total cellular protein was immunoprecipitated with anti-FLAG antibody and reacted with GST–c-jun. The bottom panel shows an anti-FLAG immunoblot. LNCaP cells have approximately sixfold-higher amount of transfected JNK1 than DU145 cells as determined by densitometry analysis. When corrected for this difference in transfected protein, MEKKΔ-DA-induced JNK activation is approximately four- to sixfold in all three cell lines. (b) Effect of JNK inhibition on MEKKΔ-DA-induced apoptosis in LNCaP cells. LNCaP cells were cotransfected with MEKKΔ-DA (0.6 μg) or Neo and pCDNA3-JBD (JNK1 inhibitor), p35 (caspase inhibitor), or vector control. (Left panel) Effect of transfected JBD on MEKKΔ-DA-induced c-jun transcriptional activity as measured by a 5X-Gal-luciferase reporter (0.4 μg) and gal4-jun (0.4 μg). (Right panel) Transfected cells were scored for apoptosis 48 h after transfection.

manner, as increasing doses of MEKKΔ-DA induced more apoptosis with a fixed amount of androgen receptor (Fig. 5a). Kinase-inactive MEKKΔ-DN failed to induce apoptosis in this assay, indicating that kinase activity is required (Fig. 5b). These data demonstrate that reconstitution of the androgen receptor pathway rescues the apoptosis defect in DU145 cells and support the hypothesis that the androgen receptor pathway is required for MEKKΔ-DA-induced apoptosis in prostate cancer cells. The fact that additional ligand is required for MEKKΔ-DA-induced apoptosis in DU145 cells but not LNCaP cells may be a consequence of androgen receptor overexpression or cell-type differences.

A corollary to the hypothesis that MEKKΔ-DA-induced apoptosis in prostate cancer cells requires functional androgen receptor is that blockade of androgen receptor signaling should protect against MEKKΔ-DA-induced apoptosis in androgen receptor-positive prostate cancer cells. We tested this hypothesis pharmacologically by using the androgen receptor antagonist casodex (49). To establish the activity of Casodex in our model, LNCaP cells were transfected with a reporter plasmid containing the promoter (P) and enhancer (E) of the androgen-dependent PSA gene fused to luciferase (PSA P/E-luc) (39). PSA is a prostate-specific, secreted kallikrein protein that is widely used as a serum marker to diagnose and monitor prostate cancer in patients (20). The promoter and enhancer both contain well-characterized androgen receptor binding sites which mediate androgen responsiveness (7, 44). Since the expression of PSA is androgen dependent, anti-androgen therapy causes a drop in PSA levels in serum, whereas relapse of androgen-independent cancer is heralded by a rise in PSA in serum. As expected, the androgen analog R1881 induced 13-fold activation of PSA P/E-luc in LNCaP cells (Fig. 6a) (39). Casodex partially inhibited PSA P/E-luc induction by ca. 40% (Fig. 6a, compare fourth and eighth columns). In the apoptosis experiments, the same concentration of Casodex partially inhibited MEKKΔ-DA-induced apoptosis by 40% and did not by

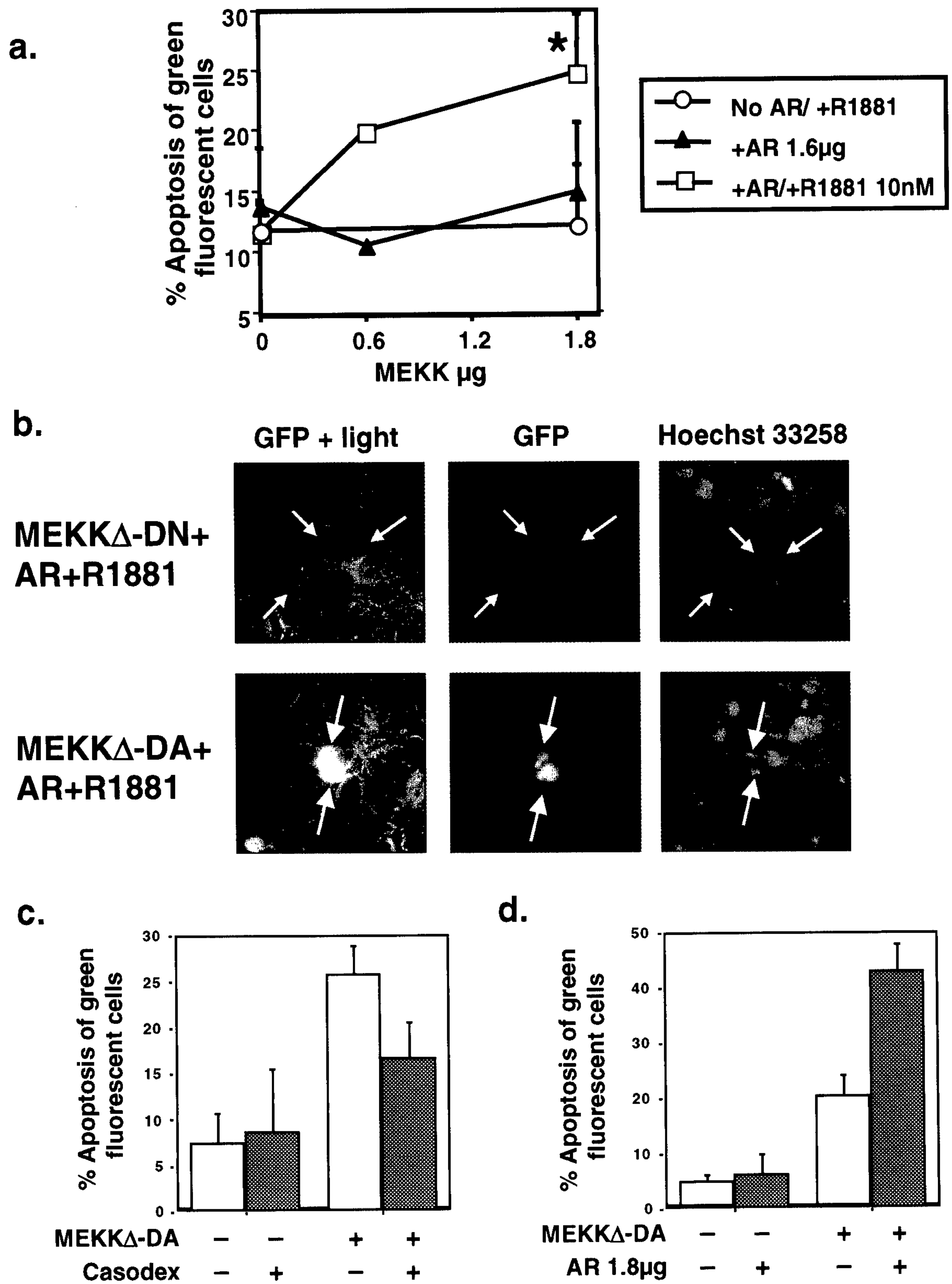

FIG. 5. Modulation of androgen receptor function alters the sensitivity of prostate cancer cells to MEKKΔ-DA-induced apoptosis. Reconstitution of the androgen receptor signaling pathway in DU145 cells. DU145 cells were cotransfected with MEKKΔ-DA, as indicated, and androgen receptor (AR) (1.8 µg) in the presence or absence of androgen R1881 (10 nM). This graph is an average of the results from eight independent experiments. The P value for the combined experiments is 0.002 as determined by the paired Student t test for MEKKΔ-DA plus androgen receptor plus R1881 versus MEKKΔ-DA plus androgen receptor. (b) Morphology of DU145 reconstituted with androgen receptor and androgen R1881 and cotransfected with MEKKΔ-DN (top row) or MEKKΔ-DA (bottom row) 48 h after transfection. White arrows indicate GFP-positive cells, and yellow arrows indicate GFP-positive cells showing chromatin condensation. (c) Effect of the androgen receptor antagonist Casodex on MEKKΔ-DA-induced apoptosis. Graph of LNCaP transfected with 0.6 µg of MEKKΔ-DA or Neo control vector and treated with the androgen receptor antagonist Casodex (10 µM) as indicated. Graph represents results of four independent experiments in which 200 green fluorescent cells were counted and scored for cytoplasmic blebbing; $P = 0.004$ as determined by the paired Student t test for MEKKΔ-DA versus MEKKΔ-DA plus Casodex. Cells were scored for apoptosis at 48 h after transfection. (d) Graph of LNCaP transfected with 0.6 µg of pCDNA3 containing MEKKΔ-DA or the empty vector and cotransfected with androgen receptor (1.8 µg) as indicated. Graph represents three independent experiments in which 200 green fluorescent cells were counted and scored for cytoplasmic blebbing at 48 h after transfection; $P = 0.01$ as determined by the paired Student t test for MEKKΔ-DA versus MEKKΔ-DA plus androgen receptor.

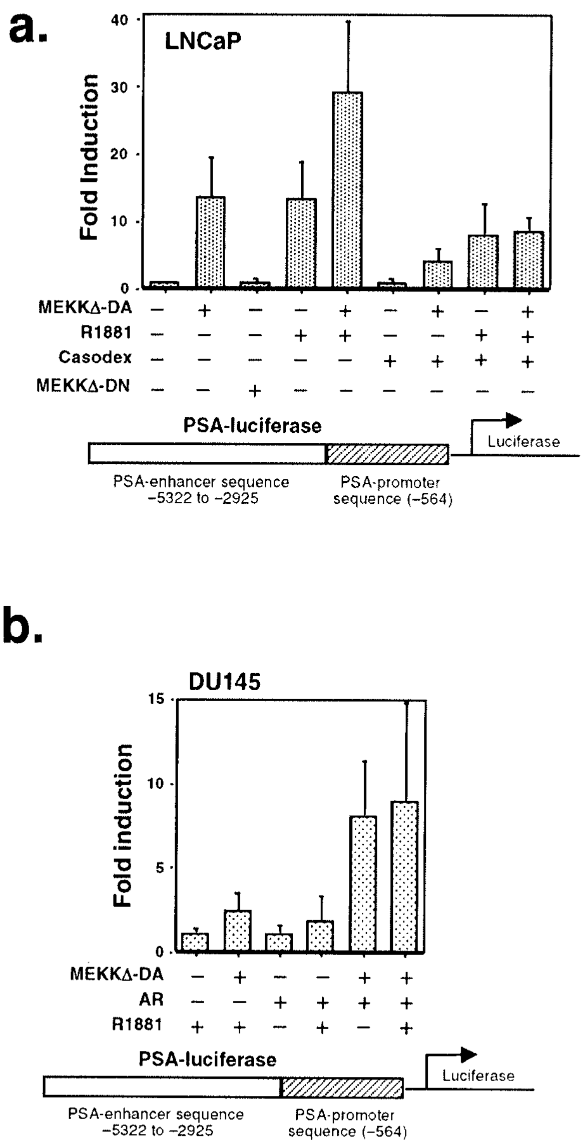

FIG. 6. MEKKΔ-DA increases the transcriptional activity of androgen receptor-regulated promoters. (a) Graph of PSA P/E-luc transcriptional activity in LNCaP cells. LNCaP cells were transfected with MEKKΔ-DA (0.6 μg) or MEKKΔ-DN or Neo control vector (3.6 μg) and cotransfected with a PSA P/E-luc reporter construct (0.4 μg). R1881 was added to a final concentration of 10 nM, and Casodex was added at a final concentration of 10 μM. This graph represents an average of six independent experiments; $P = 0.009$ as determined by the paired Student t test for MEKKΔ-DA compared to the control. (b) Graph of PSA P/E-luc transcriptional activity in DU145. DU145 were cotransfected with MEKKΔ-DA or Neo control vector (1.6 μg), androgen receptor (1.8 μg), and PSA-luc reporter (0.4 μg). R1881 was added to a final concentration of 10 nM. Luciferase activity was measured 48 h after transfection. This graph represents an average of six experiments; $P = 0.01$ for MEKKΔ-DA versus MEKKΔ-DA plus androgen receptor.

itself induce apoptosis in parental LNCaP cells (Fig. 5c). The effect of Casodex was specific for androgen receptor-positive cells because Casodex had no effect on androgen receptor-independent, MEKKΔ-DA-mediated apoptosis of HEK293 cells (data not shown). Together with the androgen receptor reconstitution experiments, these data argue for a link between the androgen receptor and the MEKKΔ-DA pathway leading to apoptosis in prostate cancer cells.

Since pharmacologic inhibition of androgen receptor function diminished MEKKΔ-DA-induced apoptosis, we reasoned that more androgen receptor expression in androgen receptor-positive cells may increase their sensitivity to MEKKΔ-DA-induced apoptosis. To test this hypothesis, LNCaP cells were transfected with wild-type androgen receptor in the presence or absence of MEKKΔ-DA (Fig. 5d). Overexpression of androgen receptor did not cause apoptosis above control levels. Coexpression of androgen receptor and MEKKΔ-DA induced apoptosis in over 40% of the cells, a significant increase compared to the expression of MEKKΔ-DA alone. These data indicate that overexpression of androgen receptor in cells with an intact androgen receptor pathway enhances MEKKΔ-DA-induced apoptosis.

One potential mechanism of MEKKΔ-DA-induced apoptosis in LNCaP cells is an alteration in the level of androgen receptor expression in androgen receptor-positive prostate cancer cells. To address this issue, androgen receptor expression was measured by immunoblot in LNCaP cells transfected with Neo or MEKKΔ-DA, LNCaP cells transfected with additional androgen receptor, and LNCaP cells stably infected with MEKKΔ-DA or control virus. No differences were seen in the endogenous expression of androgen receptor in LNCaP cells transiently or stably expressing MEKKΔ-DA (data not shown); therefore, MEKKΔ-DA does not regulate endogenous androgen receptor expression.

Activation of the MEKK1 pathway stimulates androgen-receptor regulated gene expression. Activation of the tyrosine kinase receptors for KGF and IGF-1 or protein kinase A activation increases androgen receptor-mediated gene transcription in the absence of androgen, suggesting cross-talk with the androgen receptor pathway (11, 38). Because MEKKΔ-DA induces apoptosis in prostate cancer cells in an androgen receptor-dependent fashion, we hypothesized that MEKK1 signaling may also affect androgen receptor-mediated gene transcription. To test this hypothesis, LNCaP cells were transfected with the PSA P/E-luc reporter plasmid described above and cotransfected with MEKKΔ-DA in the presence or absence of androgen. Experiments were performed in medium containing charcoal-stripped serum to exclude potential effects of steroid hormones in FCS. MEKKΔ-DA activated the reporter 14-fold in the absence of androgen (Fig. 6a). Thus, the expression of MEKKΔ-DA results in androgen-independent PSA transcriptional activation that is similar in magnitude to the treatment of cells with androgen. The combination of MEKKΔ-DA and androgen led to further activation of PSA P/E-luc transcription (average fold induction of 30). To test whether transcriptional activation of PSA P/E-luc by MEKKΔ-DA required its kinase activity, LNCaP cells were transiently transfected with kinase inactive MEKKΔ-DN. MEKKΔ-DN had no effect on transcriptional activation, demonstrating that the kinase activity of MEKK1 is required for this effect.

We explored the role of the androgen receptor in MEKKΔ-DA induction of PSA transcriptional activity by using two complementary strategies. First, we asked if the androgen receptor antagonist Casodex inhibited MEKKΔ-DA activation of PSA P/E-luc in LNCaP cells. PSA P/E-luc activity in cells cotransfected with MEKKΔ-DA was reduced by Casodex from 14-fold to 4-fold (Fig. 6a, compare second and seventh columns). These results suggest that ligand-independent activation of the PSA promoter-enhancer by MEKKΔ-DA is mediated by the androgen receptor. To confirm this hypothesis we performed further experiments in androgen receptor-negative DU145 cells. The absence of endogenous androgen receptor expression in DU145 allowed us to study the effect of MEKKΔ-DA on the PSA promoter-enhancer in the presence or absence of transfected androgen receptor. Androgen in-

duced activation of PSA P/E-luc a modest twofold when androgen receptor was included in the transfection (Fig. 6b), a result consistent with previous reports (55). Transfection of MEKKΔ-DA in the absence of androgen receptor also activated PSA P/E-luc twofold. However, the combination of androgen receptor and MEKKΔ-DA resulted in an average eightfold activation which was not enhanced further by androgen. In conjunction with the Casodex experiments, these data indicate that the effect of MEKKΔ-DA on PSA transcriptional activity requires the androgen receptor.

In addition to androgen receptor binding sites (AREs), the PSA promoter contains other transcription factor binding motifs, such as AP-1 recognition sites (46). Since MEKKΔ-DA activates transcription factors such as AP-1, a potential mechanism for cross-talk between MEKKΔ-DA signaling and the androgen receptor pathway is through cooperative effects between AP-1 sites and AREs in the PSA promoter and enhancer (8). Alternatively, MEKKΔ-DA-mediated induction of the PSA promoter may function solely through activation of the androgen receptor. We addressed this issue by examining the effect of MEKKΔ-DA on an artificial promoter consisting of four AREs multimerized upstream of the E4-CAT reporter gene (4X-ARE/E4-CAT) in DU145 cells. In the absence of transfected androgen receptor, neither MEKKΔ-DA nor androgen activated the 4X-ARE/E4-CAT reporter (Fig. 7a). Androgen activated the 4X-ARE/E4-CAT reporter 38-fold after reconstitution with androgen receptor. Cotransfection of androgen receptor and MEKKΔ-DA enhanced activation of the reporter from 38-fold to 170-fold in the presence of ligand. These effects are specific to AREs because MEKKΔ-DA had no effect on the parental E4-CAT reporter pBXG0, which lacks the AREs (Fig. 7b, lanes 1 and 2) or on the pZRE5-E4-CAT reporter in which the ARE sites were replaced with sites for the Epstein-Barr virus (EBV) transcription factor ZEBRA (Fig. 7b, lanes 3 and 4). These data indicate that the effect of MEKKΔ-DA on androgen receptor-mediated gene activation can be mediated through AREs in the absence of AP-1 sites. In contrast to the ligand-independent effects of MEKKΔ-DA in the context of the natural PSA promoter, ligand binding of androgen to androgen receptor is required to mediate the effect of MEKKΔ-DA on an artificial template containing only AREs. These differences may be a consequence of additional, ARE-independent effects of the PSA promoter. Alternatively, the effects of MEKK1 on these reporters, as well as apoptosis, may not be strictly correlated.

One potential explanation for the enhanced transcriptional activation of androgen receptor-regulated genes by MEKKΔ-DA is that MEKKΔ-DA is having nonspecific effects on the general transcription machinery. To test this possibility, we examined the effects of MEKKΔ-DA on another reporter system based on the EBV-derived transcription factor ZEBRA. This system is ideal for addressing the specificity of MEKKΔ-DA-induced transcriptional activation because the relationship between ZEBRA, its binding to core promoter elements, and the activation of the general transcription machinery have been carefully characterized (31). If MEKKΔ-DA acts nonspecifically, we would expect enhanced activation of pZRE5-E4-CAT in the presence of MEKKΔ-DA. However, MEKKΔ-DA had no effect on ZEBRA-mediated induction of pZRE5-E4-CAT (Fig. 7b, lanes 5 and 6). These data argue for specificity in the effects of MEKKΔ-DA on androgen receptor-mediated transcription.

Based on our finding that MEKKΔ-DA-induced apoptosis of prostate cancer cells is dependent on androgen receptor signaling and that MEKKΔ-DA activates androgen receptor-dependent transcription, we sought to determine whether the

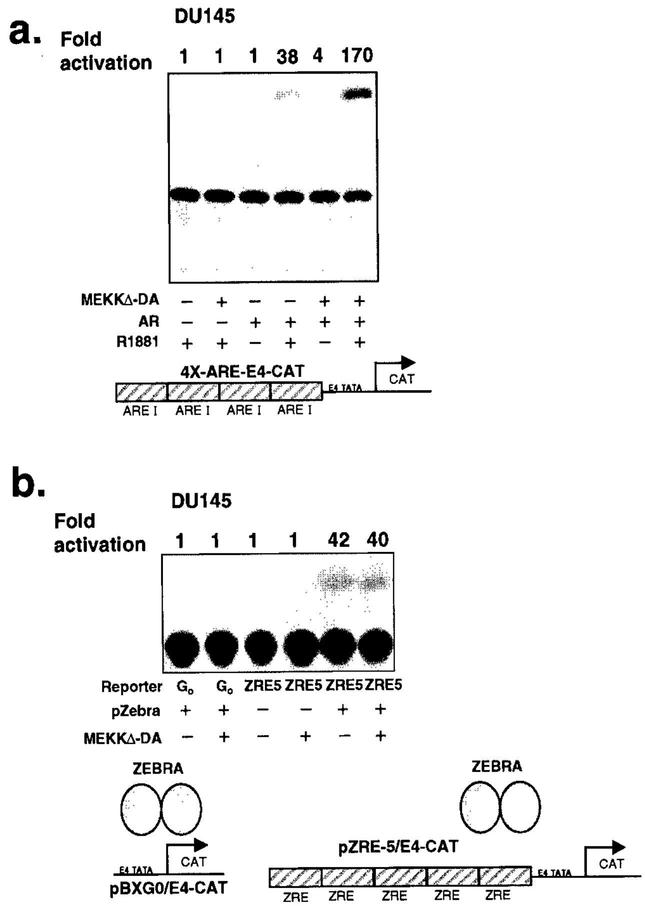

FIG. 7. MEKKΔ-DA specifically increases the transcriptional activity of androgen receptor on a minimal promoter element. (a) Effect of MEKKΔ-DA on transcriptional activation of a promoter consisting of pure androgen response elements in DU145. A promoter consisting of four multimerized androgen response elements, 4X-ARE/E4-CAT (0.4 μg) was transfected into DU145 as in Fig. 6b. CAT production was analyzed by ELISA as described in Materials and Methods and by conventional CAT assay. ImageQuant software was used to analyze phosphorimager data for the conventional CAT assay. This is one representative experiment of four total. (b) Effect of MEKKΔ-DA on the transcriptional activation of a promoter consisting of ZEBRA response elements in DU145 cells. For these experiments, DU145 stably expressing androgen receptor or Neo-infected cells were transfected with the vectors as indicated: 0.8 μg of reporter plasmid, 0.8 μg of ZEBRA transcription factor, and 2.4 μg of MEKKΔ-DA or Neo vector control. The data shown were obtained with androgen receptor-expressing DU145 cells.

MEKK signaling pathway plays a role in ligand-mediated activation of the androgen receptor in prostate cells. To test this possibility, we measured the effects of the dominant negative mutant, MEKKΔ-DN, on androgen-regulated gene expression (36). As expected, MEKKΔ-DA activated the PSA P/E-luc reporter in LNCaP cells. To validate the ability of MEKKΔ-DN to function as an MEKK antagonist, LNCaP cells were cotransfected with MEKKΔ-DA and MEKKΔ-DN (Fig. 8, left panel). MEKKΔ-DN inhibited MEKKΔ-DA-induced transcriptional activation of the PSA P/E-luc reporter between 50 and 75%. We then tested the ability of MEKKΔ-DN to inhibit androgen-mediated PSA P/E-luc activation. In four independent experiments, MEKKΔ-DN inhibited R1881-induced transcriptional activation of the PSA P/E-luc reporter in a dose-dependent fashion (Fig. 8, right panel). When similar experiments were performed with the 4X-ARE-CAT reporter in DU145 cells, we failed to see significant effects of

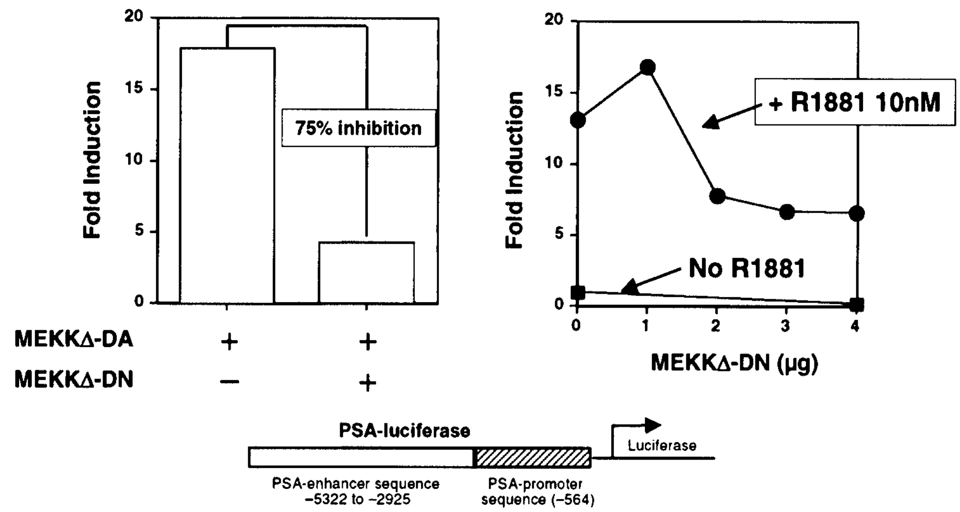

FIG. 8. Expression of MEKKΔ-DN inhibits androgen-mediated activation of PSA P/E-luc. (Left panel) Effect of MEKKΔ-DN on MEKKΔ-DA-induced PSA P/E-luc activity. LNCaP cells were transfected with MEKKΔ-DA (0.6 μg) and cotransfected with 3.6 μg (6:1 ratio) of MEKKΔ-DN as indicated. This is one representative experiment of three total, all with similar results. (Right panel) Effect of increasing amounts of MEKKΔ-DN on R1881-induced PSA P/E-luc activity. LNCaP cells were transfected with increasing amounts of MEKKΔ-DN as indicated in the presence of R1881 (10 nM). This is one representative experiment of four total, all with similar results.

MEKKΔ-DN on R1881-mediated activation of this reporter (data not shown). Therefore, the inhibitory effects of MEKKΔ-DN on the PSA P/E-luc reporter may be related to *cis*-acting elements which influence the outcome of androgen receptor activation in the context of a natural promoter.

DISCUSSION

Previous work on MEKK1 function has defined a role for this pathway in signaling involving the stress response (6, 13), NF-κB activation (30, 56), and integrin receptor engagement (5, 18). Results presented here provide evidence of a role in androgen receptor signaling in prostate cells. At a transcriptional level a constitutively active allele of MEKK1 stimulates natural and artificial androgen-responsive promoter templates in an androgen receptor-dependent fashion. In addition, transcriptional activation of the androgen receptor by androgen is impaired when a dominant negative mutant of MEKK1 is coexpressed. Taken together, these results suggest that the MEKK1 pathway plays a role in modulating the transcriptional response of the androgen receptor to ligands. Importantly, this cross-talk extends beyond the level of transcription to the biological response of cells to MEKK1 signaling. Consistent with previous reports in fibroblasts and T cells (16, 25), constitutive activation of MEKK1 induces apoptosis in prostate cancer cells. However, the apoptotic effect in prostate cells occurs only when the androgen receptor signaling pathway is intact. The evidence supporting this conclusion are the correlation of MEKKΔ-DA-induced apoptosis with androgen receptor expression, the ability of androgen receptor expression to restore the ability of MEKKΔ-DA to induce apoptosis in androgen receptor-negative prostate cancer cells, the potentiation of MEKKΔ-DA-induced apoptosis by overexpression of androgen receptor in androgen receptor-positive prostate cancer cells, and the partial inhibition of MEKKΔ-DA-induced apoptosis by androgen receptor blockade. In summary, our results establish a pattern of cross-talk between the MEKK1 and the androgen receptor pathways in prostate cells at a transcriptional and biological level.

The discovery of an interaction between the androgen receptor and MEKK1 signaling pathways adds to growing evidence that a number of different tyrosine and serine-threonine kinases can affect the function of steroid hormone receptors (4, 10, 11, 27). The molecular basis for each distinct example of cross-talk remains unknown and is the focus of much current research. A better understanding of this mechanism is likely to have important implications for hormone receptor regulation in cancer cells. In the case of MEKK1, its large size (196 kDa) and known ability to assemble in multiprotein complexes (12, 30, 56), as well as to interact with an array of signaling proteins (15, 54, 56), raise the possibility of a multiprotein signaling complex involving the androgen receptor in prostate cells. Alternatively, MEKK1 may activate a signaling cascade that indirectly leads to posttranslational modifications of the androgen receptor which affect its function, a possibility analogous to reported effects of the ERK pathway on the estrogen receptor (4, 24, 27, 58). It is also possible that MEKK1 affects coactivators, such as ARA-70 and GRIP-1 (22, 55), rather than androgen receptor itself or that it functions through transcription factors, such as c-jun (3, 43, 52), which act cooperatively with the androgen receptor to facilitate gene expression. More research is needed to sort through these various models.

MEKK1-induced apoptosis is known to occur in non-androgen-receptor-expressing cells such as fibroblasts (25), human embryonal kidney cells, and fibrosarcoma cells (51). In some settings, UV irradiation, chemotherapy, or tumor necrosis factor α are required to elicit the apoptotic phenotype, suggesting that MEKK1-induced apoptosis may require additional signals to initiate the apoptotic cascade. Our results would argue that androgen receptor signaling may be such a signal in prostate cells. This idea may seem paradoxical since androgen confers a survival and/or proliferative signal in prostate secretory epithelial cells. However, excess androgen receptor signaling in certain settings is detrimental to cell growth and survival. For example, androgen inhibits the growth of androgen receptor-positive LNCaP cells at high concentrations in vitro (48), and androgen receptor-negative PC3 cells transfected with a constitutively active androgen receptor have delayed growth compared with mock-transfected cells (33). Consistent with these reports, we find that excess androgen induces low levels of

apoptosis in LNCaP cells in vitro (1). We hypothesize that excess stimulation of the androgen receptor signaling pathway, through MEKK1 activation or excess androgen, can lead to apoptosis of prostate cancer cells. This scenario is consistent with more extensively characterized signaling molecules such as the glucocorticoid receptor (17, 21) and c-Myc (1a, 45), which can induce either cell cycle progression or apoptosis in distinct cellular or environmental contexts.

In addition to the implications for hormone receptor signaling, our results offer potential insight into the mechanisms of prostate cancer progression. Anti-androgen therapy is the primary clinical treatment of metastatic prostate cancer and induces temporary remissions in the majority of patients. Eventually, prostate cancer cells regrow despite anti-androgen therapy, and the majority continue to express androgen receptor (40) and androgen-regulated genes such as PSA. This phenotype suggests that alternative, androgen-independent signaling pathways are utilized to activate the androgen receptor in these cells. Our observation that MEKK1 can substitute for androgen in androgen receptor-dependent transcription raises the possibility that this pathway may function in the progression to androgen independence. Further experiments with animal models and clinical material are required to address this hypothesis. Alternatively, the androgen receptor-dependent apoptotic function of activated MEKK1 in prostate cells might provide a therapeutic opportunity in androgen-independent prostate cancers. Because of its ability to sensitize cells to genotoxic stress (25, 51), expression of MEKK1 may be considered a strategy for cancer gene therapy.

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Evidence for Clonal Outgrowth of Androgen-independent Prostate Cancer Cells from Androgen-dependent Tumors through a Two-Step Process[1]

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ABSTRACT

Prostate cancers require androgen for growth but progress to an androgen-independent stage under the selective pressure of androgen ablation therapy. Here we describe a novel human prostate cancer xenograft (LAPC-9) propagated by serial passage in male severe combined immunodeficient mice that expresses prostate-specific antigen and wild-type androgen receptor. In response to castration, LAPC-9 cells undergo growth arrest and persist in a dormant, androgen-responsive state for at least 6 months. After prolonged periods of androgen deprivation, spontaneous androgen-independent outgrowths develop. Thus, prostate cancers progress to androgen independence through two distinct stages, initially escaping dependence on androgen for survival and, subsequently, for growth. Through the use of serial dilution and fluctuation analysis, we provide evidence that the latter stage of androgen independence results from clonal expansion of androgen-independent cells that are present at a frequency of about 1 per 10^5/10^6 androgen-dependent cells. We conclude that prostate cancers contain heterogeneous mixtures of cells that vary in their dependence on androgen for growth and survival and that treatment with antiandrogen therapy provides selective pressure and alters the relative frequency of these cells, thereby leading to outgrowths of androgen-independent cancers.

INTRODUCTION

Androgen withdrawal leads to apoptosis of the secretory epithelium and growth arrest of the basal epithelium in the normal prostate (1). Androgen replacement stimulates the repopulation of secretory epithelium through induction of a differentiation program in a subset of basal cells (2, 3). Thus, basal cells are androgen responsive but are not dependent on androgen for survival, whereas secretory cells require androgen to avoid apoptotic cell death. Most prostate cancers are considered androgen dependent, based on the high response rate of these tumors to antiandrogen therapy. The mechanism for the clinical response to androgen withdrawal therapy is not clearly defined but is likely to result from a combination of tumor cell death through induction of apoptosis as well as growth arrest (4–6). Eventually, prostate cancers will resume growth despite antiandrogen therapy, at which point the tumors are termed androgen independent or hormone refractory.

At a molecular level, androgen-independent progression has been associated with mutations or amplification of the androgen receptor gene (7–12) and activation of intracellular signal transduction pathways that stimulate the androgen receptor (13–15). These observations have led to the concept that androgen-independent prostate cancers have reactivated the androgen receptor pathway by a ligand-independent mechanism (16, 17). Interestingly, comparative gene expression studies indicate that some androgen-independent cancers may express genes normally restricted to the basal epithelium of normal prostate (18, 19). In contrast, their androgen-dependent counterparts have a gene expression profile more typical of differentiated, secretory epithelial cells. These findings raise questions about the cell of origin in prostate cancer and are consistent with the notion that androgen-independent cancers represent the outgrowth of a rare, preexisting subclone of tumor cells with basal cell characteristics. This concept has been supported previously by fluctuation analysis in the rat Dunning system (20–23). Additionally, recent evidence in the transgenic mouse TRAMP model suggests that androgen-independent prostate cancer cells occur very early in the progression of disease (24). Alternative possibilities are that androgen-dependent prostate cancer cells develop secondary genetic mutations that allow androgen-independent growth or that populations of androgen-dependent cells adapt to the altered hormonal environment caused by androgen deprivation, as reported in the Shionogi mouse mammary carcinoma model (25–27). Although these questions have been well studied in rodents, it has been difficult to distinguish between these concepts in human prostate cancer cells, largely because appropriate models to study this question have been lacking.

Our laboratory previously reported a human prostate cancer xenograft called LAPC[3]-4, which progresses from androgen dependence to androgen independence in SCID mice in response to castration (28). Here we describe a new prostate cancer xenograft, LAPC-9, that also requires androgen for growth, synthesizes PSA, and expresses a nonmutant androgen receptor. Through kinetic analysis of *in vivo* proliferation and cell death, we show that a small fraction of the cells in LAPC-9 tumors die by apoptosis in response to castration, whereas the majority withdraw from the cell cycle. These cells remain in a dormant yet viable state and respond rapidly when reexposed to androgen by reentering the cell cycle and resuming tumor growth, even after 6 months of androgen deprivation. After longer intervals, some LAPC-9 tumors resume growth as androgen-independent cancers.

The availability of two androgen-dependent xenografts that develop androgen independence after castration provides an opportunity to investigate the cellular basis of this progression in an experimental model. Through the use of serial dilution studies and fluctuation analysis, we show that injection of as few as 10 cells will consistently lead to tumor formation in intact male mice, but only a fraction of such injections will produce tumors when implanted in castrated mice. Fluctuation analysis was used originally to provide evidence that the emergence of bacterial strains resistant to bacteriophage lysis is a consequence of preexisting genetic mutations in the bacteria rather than an adaptive response to altered nutrients (29). Our results are consistent with the hypothesis that hormone-refractory cancer evolves through clonal outgrowth of a small number of androgen-independent

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[3] The abbreviations used are: LAPC, Los Angeles prostate cancer; SCID, severe combined immunodeficient; PSA, prostate-specific antigen; DHT, dihydrotestosterone; PrEGM, prostate epithelial-specific growth media.

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tumor cells that are preexisting or develop at a low frequency due to secondary genetic mutations. We propose that prostate cancers contain a mixture of cells that differ in their need for androgen as a growth or survival factor and that antiandrogen therapy gives androgen-independent cells a selective growth advantage that results in outgrowths of hormone-refractory tumors.

MATERIALS AND METHODS

Development of the LAPC-9 Xenograft. LAPC-9 was derived as described previously for LAPC-4 (28). After obtaining informed consent, clinical material was obtained at the time of surgery from the femoral metastasis of a patient who had disease progression while receiving hormone ablation therapy. The tissue was minced and implanted with 200 μl of Matrigel (Collaborative Research, Bedford, MA) s.c. into several male SCID mice under methoxyflurane anesthesia. After initial tumor formation, tumors were harvested, minced, and reimplanted with Matrigel into male SCID mice. Androgen ablation was achieved by surgical castration under anesthesia. Androgen replacement after castration was achieved using implantable sustained release DHT pellets (12.5 mg/90-day release; Innovative Research of America, Sarasota, FL).

Androgen Receptor Sequencing. Individual pairs of oligonucleotides were used to amplify segments of the androgen receptor gene as described previously (30). The PCR product was cloned into pZero Blunt (Invitrogen, San Diego, CA) according to the manufacturer's protocol and sequenced using automated methods through the UCLA DNA sequencing facility.

Preparation of Single-Cell Xenograft Suspensions. Tumors were dissociated into single-cell suspensions by enzymatic digestion with modifications to a protocol described previously (31). Briefly, LAPC xenografts were harvested using sterile technique from the flanks of SCID mice. Tumors were minced to 1-mm^3 pieces in serum-free Iscove's medium on ice. This tissue was washed twice with Iscove's medium, and then incubated in a 1% solution of Pronase E (EM Science, Gibbstown, NJ) in Iscove's for 18 min at room temperature using 10 times the original tumor volume. Tissue was washed twice in Iscove's medium, filtered though sterile 200-μm nylon mesh (Biodesign, Inc. of New York, Carmel, NY), and plated overnight at 37°C in serum-free PrEGM (Clonetics, San Diego, CA) containing Fungizone. The next day, tissue was disaggregated again by pipetting, refiltered through nylon mesh, and replated in PrEGM overnight to obtain a homogenous single-cell preparation. On the third day, the cells were washed once in PrEGM, counted, serially diluted in PrEGM, then injected s.c. into both flanks using a 25-gauge needle with 100 μl of Matrigel extracellular matrix. For tissue chunks, 2-mm^3 chunks of tissue were implanted using a trocar as described previously (28). Tumors were monitored by palpation every 1–2 weeks and measured in three orientations using calipers.

Protein Expression Studies. PSA levels in mouse serum were determined by ELISA (American Qualex, San Clemente, CA) according to product literature and calibrated to the PSA controls provided. Tissues were fixed for 4 h in 10% neutral buffered formalin and then embedded in paraffin for histological sectioning. Antigen retrieval was performed using a commercial steamer and incubation in a 0.01 M citrate buffer (pH 6). Serial sections were incubated with monoclonal antibody to PSA diluted 1:3000 in PBS (DAKO Corp., Carpinteria, CA) or MIB-1 antibody (against Ki-67) diluted 1:60 (Immunotech, Westbrook, ME). Slides were then incubated sequentially with peroxidase-conjugated rabbit anti-mouse antibodies, peroxidase-conjugated swine anti-rabbit antibodies, and peroxidase-conjugated rabbit anti-swine (DAKO Corp.). Antibody localization was performed using the diaminobenzidene reaction, and slides were counterstained with hematoxylin. Terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling staining was performed using the ApopTag Plus kit (Intergen, Purchase, NY) according to the manufacturer's instructions.

RESULTS

Characterization of a New PSA-positive, Androgen-dependent Human Prostate Cancer Xenograft. To expand the spectrum of prostate cancer phenotypes represented by cell lines and xenografts, we have continued our efforts to develop new human prostate cancer xenografts in SCID mice (28). Similar to the previously reported xenografts LAPC-3 and LAPC-4, LAPC-9 was developed by implanting prostate cancer cells obtained at the time of surgery directly into SCID mice. LAPC-9 has been maintained for >20 passages in male SCID mice over a 2-year period without administering supplemental testosterone. ELISA analysis of serum from mice bearing LAPC-9 xenografts (Fig. 1) demonstrated expression of the androgen-dependent *PSA* gene, indicating that LAPC-9 tumors are of human prostatic origin and have an intact androgen receptor signaling pathway. As expected, this conclusion was confirmed by immunoblot analysis showing expression of androgen receptor protein in LAPC-9 cells (data not shown).

F1

Although mutations in the androgen receptor gene occur in some prostate cancers (7–11), most clinical samples appear to express wild-type androgen receptor. To determine the status of the androgen receptor in LAPC-9, we sequenced the coding regions of the gene using genomic DNA. No mutations were found based on sequence analysis of PCR products obtained by amplification of exons 1–8 using intron-based primers and genomic DNA as template.

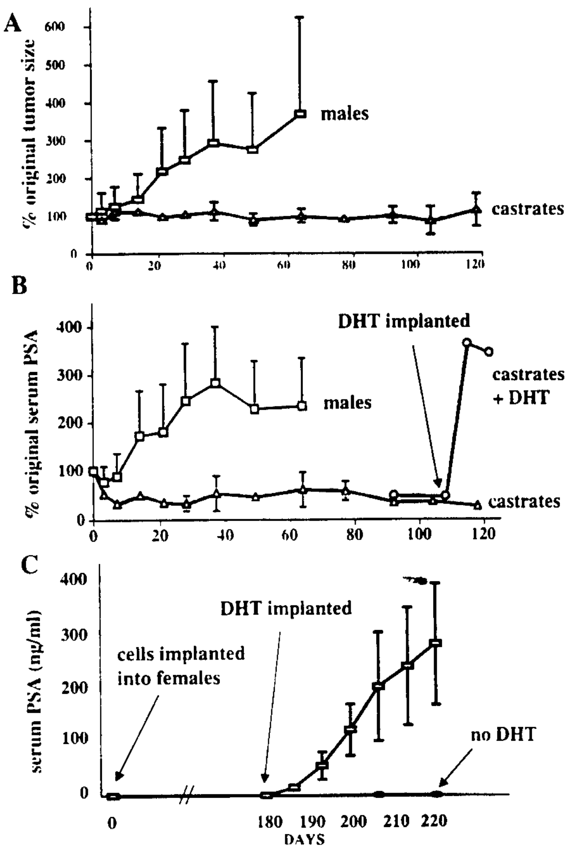

Fig. 1. *In vivo* growth characteristics of LAPC-9 tumors in the presence or absence of androgen. LAPC-9 cells were harvested from intact male mice and prepared as described in "Materials and Methods." *A* and *B*, male SCID mice were implanted with 10^6 LAPC-9 cells, and tumors were allowed to grow to 0.5 cm^3 over about 5 weeks. Mice were then castrated ($n = 4$), and tumor size (*A*) and serum PSA (*B*) was monitored and compared with uncastrated controls ($n = 5$) at the time points noted. Values are normalized to 100% on day 0, which is the day of castration. After 110 days, DHT pellets were reimplanted into castrated animals, and the response in serum PSA is shown in *B*. *C*, 10^4 LAPC-9 cells were injected s.c. into the flanks of female mice. After 6 months, DHT pellets were implanted, and serum PSA was measured weekly. Data points are serum PSA in ng/ml are expressed as means ($n = 5$); *bars*, SD.

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To examine the role of androgen in LAPC-9 tumor growth, we set up a series of experiments in which tumors were established s.c., subjected to androgen deprivation, and in some cases, reexposed to androgen 3–6 months later. We implanted LAPC-9 cells into nine intact male mice, allowed tumors to develop, then castrated four animals. In uncastrated control mice, tumor size (Fig. 1*A*) and serum PSA (Fig. 1*B*) continued to rise for 60 days until the mice were euthanized because of tumor burden. In castrated animals, the serum PSA fell by 70% after 7 days and remained at a low but detectable level for the duration of the experiment (120 days). Tumor size showed little decrease after castration and remained unchanged throughout the experiment. These data indicate that LAPC-9 cells require androgen for continued growth.

Secretory epithelial cells in the normal prostate gland as well as some prostate cancer xenografts undergo apoptosis in response to androgen withdrawal (32–34). We used standard immunohistochemical markers of cell growth and cell death at various time points after castration to determine the effect of androgen withdrawal on these parameters in LAPC-9 tumors. In the presence of androgen, a high fraction of LAPC-9 cells expressed the proliferation marker protein Ki-67, indicating a high growth fraction (Fig. 2, *upper left panel*, day 0). After castration, the number of Ki-67-positive cells fell more than 10-fold over 7 days and remained low throughout the period of androgen deprivation (Fig. 2, 110 days). A very small fraction (~1%) of cells were terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling-positive at all time points before and after castration, indicating a low rate of apoptosis (data not shown). Therefore, the primary consequence of androgen deprivation in the LAPC-9 tumor model is a decrease in cell proliferation, a finding consistent with the lack of significant regression in tumor size (Fig. 1*A*).

F2

LAPC-9 Cells Remain Dormant But Are Androgen Responsive after Prolonged Periods of Androgen Ablation. The fact that a significant fraction of LAPC-9 tumor cells fail to die after castration raises the possibility that these residual cells are androgen independent. Alternatively, they may no longer require androgen for survival but remain dependent on androgen for growth. To examine this possibility, we asked whether LAPC-9 cells remain androgen responsive after prolonged periods of androgen deprivation. LAPC-9 tumors were established in intact male mice, and then the mice were castrated. PSA levels fell by 70% and reached a plateau phase, as in Fig. 1*B*. One hundred ten days after castration, DHT pellets were implanted s.c. in a cohort of animals to reexpose the residual tumor cells to androgen. Within 14 days, serum PSA levels rose 30-fold in the DHT-treated group (Fig. 1*B*) but not in untreated animals. Histological analysis showed a 15- to 20-fold increase in the number of Ki-67-positive cells within 7 days (Fig. 2). This was followed by a rapid increase in tumor size with kinetics comparable with tumors implanted into intact males (data not shown). These results indicate that LAPC-9 tumors remain androgen responsive after prolonged periods of androgen deprivation.

One explanation for the androgen-independent survival of LAPC-9 tumors is the presence of an established vasculature that might provide essential survival factors in the absence of androgen. Alternatively, LAPC-9 cells may be completely independent of androgen for survival, yet require androgen for growth. To distinguish between these possibilities, we injected LAPC-9 cells that had been disaggregated into a single-cell suspension into the flanks of female mice at doses that fail to give rise to tumors after 1 year, reasoning that any tumors that formed after reexposure to androgen could not be explained by tumor vasculature. Six months after injecting 10^4 cells s.c., no tumors were present, and serum PSA levels were undetectable. We then implanted DHT pellets and monitored serum PSA levels and tumor formation. Within 7 days, serum PSA levels rose from undetectable levels to a mean of 13.2 ng/ml (Fig. 1*C*). After 14 days, PSA levels reached 51.4 ng/ml, and tumors were palpable at the site where cells had been injected s.c. 6 months earlier. By 28 days, PSA levels were >200 ng/ml, and the mice were sacrificed shortly thereafter because of tumor burden. These results indicate that single-cell suspensions of LAPC-9 cells implanted directly into an androgen-deprived environment remain viable but do not proliferate. We conclude that androgen confers a potent growth signal in the LAPC-9 prostate cancer model but is not required for survival.

Isolation of Androgen-independent LAPC-9 Sublines. The previously developed androgen-dependent LAPC-4 xenograft, which grows reproducibly in intact male mice within 4 weeks after trocar

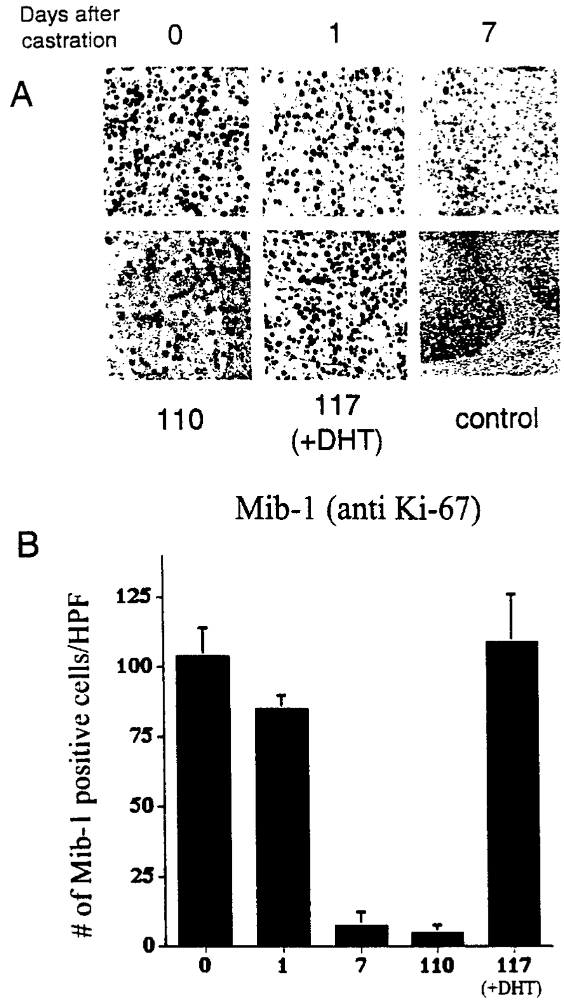

Fig. 2. Histological response of LAPC9 AD tumors to castration. LAPC-9 cells were implanted into intact male mice, and tumors were allowed to grow to 0.5 cm³. Mice were then castrated and tumors harvested at the time points listed. Tissue was prepared as described in "Materials and Methods," and sections were stained with the anti-Ki-67 antibody Mib-1. *A*, photomicrographs are shown from day 0, 1, 7, and 110 after castration and at day 117 (7 days after reexposure to DHT). *control*, a preparation of human lymph node to validate the performance of the antibody. *B*, average number of Mib-1-positive cells per high power field (×180) as determined by counting six independent regions of the slide. *Bars*, SD.

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implantation of minced tumor tissue, will form tumors spontaneously in castrated male mice after 13–16 weeks without the readdition of androgen. We have demonstrated previously that these LAPC-4 tumors derived from castrated animals (called LAPC-4 AI) are androgen independent because they grow at comparable rates when passaged into intact or castrated male mice (28). We performed a similar experiment with LAPC-9 and found that trocar implants in intact male mice formed tumors in 5 weeks, whereas similar implants in castrated males formed tumors after 26 weeks (Table 1). Of note, these tumors (called LAPC-9 AI) regrew within 4 weeks when passaged into female mice, confirming that these sublines are androgen independent for growth. Therefore, sublines of LAPC-4 and LAPC-9 can be derived that are hormone refractory in that they do not require androgen for growth or survival.

T1

Evidence for Clonally Derived, Androgen-independent Cells in Androgen-dependent Xenografts. On the basis of studies of androgen-responsive Shionogi mouse mammary carcinoma cells, it has been argued that conversion to androgen independence is an adaptive process that occurs in response to androgen ablation therapy (25–27). This argument is based on the observation that the frequency of androgen-independent cells in Shionogi tumors that regress after castration is much lower than in recurrent hormone refractory Shionogi tumors, implying that androgen-dependent cells adapt to an altered hormonal environment. Alternatively, it has been proposed from studies of the Dunning rat prostate cancer model that these tumors are heterogeneous and that androgen-independent cells undergo clonal expansion in the setting of antiandrogen therapy (20, 22, 23). These cells could preexist at low frequency in the original androgen-dependent tumor, as suggested by studies in the TRAMP model (24), or develop as a consequence of secondary mutations or epigenetic changes arising during androgen ablation therapy. Because the Shionogi, Dunning, and TRAMP models use rodent carcinoma cells rather than human prostate cancer cells, we examined these concepts in LAPC-4 and LAPC-9 cells. If the clonal expansion hypothesis is true, it should be possible to subdivide a population of androgen-dependent tumor cells into pools, some of which will contain androgen-independent cells and some of which will not, analogous to the fluctuation analysis strategies used to demonstrate preexisting genetic resistance of bacteria to bacteriophage lysis (29) and to show clonal selection in the Dunning system (20). If the adaptive model is correct, then all pools should give rise to androgen-independent tumors. The size of the pools required to dilute out androgen-independent cells will depend on the frequency of these cells in the original androgen-dependent population. Because LAPC-4 and LAPC-9 give rise to androgen-independent sublines with different kinetics (Table 1), we reasoned that the frequency of androgen-independent cells may be different in these two lines.

We performed a serial dilution analysis of androgen-dependent LAPC-4 and LAPC-9 cells in intact male mice. The purpose was to define the smallest number of cells capable of forming a tumor in the presence of androgen to establish the limits of the model. For both xenografts, the latency for tumor formation was strictly related to the

Table 1 *LAPC tumor latency in male and castrate male mice*

LAPC-4 or LAPC-9 tumors were harvested and prepared as in "Materials and Methods." Minced tissue was mixed with Matrigel and implanted by trocar s.c. into male or castrated male mice. The average number of weeks to tumor formation (>0.5 cm) is shown for each condition. The number of tumors and sample sizes are shown in parentheses.

	LAPC4	LAPC9
AD in males	28 days (6/6)	35 days (6/6)
AD in castrates	98 days (5/6)	182 days (4/6)
AI in castrates	28 days (6/6)	28 days (6/6)

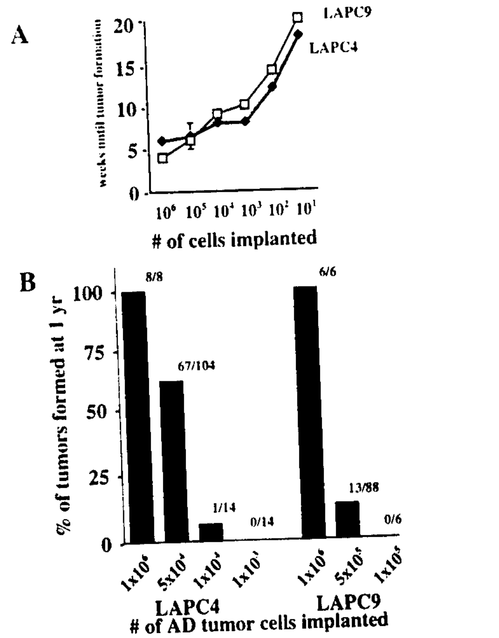

Fig. 3. Limiting dilution and fluctuation analysis of LAPC-4 and LAPC-9 xenografts. LAPC-9 and LAPC-4 cells were harvested from intact male mice and prepared as described in "Materials and Methods." *A*, cells were implanted s.c. into intact male mice at the cell dose specified (n = 6–14 mice at each cell dose), and tumors were measured by calipers weekly. Data points represent the time point at which 50% of implantations formed tumors >0.5 cm in all dimensions. *B*, LAPC-4 or LAPC-9 cells were implanted s.c. into female mice and monitored weekly. Tumor formation was positive if a tumor was >0.5 cm in all dimensions. Data points represent the percentage of implantations that resulted in tumor formation at 1 year. Absolute number of tumors formed and sample sizes are shown next to each column.

F3

dose of cells injected, and as few as 10 cells was sufficient to form a tumor (Fig. 3*A*). These results establish a high cloning efficiency for LAPC-4 and LAPC-9 cells when isolated from androgen-dependent xenografts and implanted in intact males.

To determine whether androgen-independent cells are present in these tumors and to define their frequency, androgen-dependent LAPC-4 and LAPC-9 tumors were divided into pools ranging in size from 10 to 1×10^6 cells and injected into female mice (Fig. 3*B*). For LAPC-4, all of the animals injected with 10^6 cells developed tumors, indicating that the frequency of androgen-independent cells is at least 1 per million. However, at a pool size of 5×10^4 cells, 67 of 104 (64%) female animals developed tumors. Only one of 14 animals (7%) developed a tumor at a pool size of 1×10^4, and no tumors were observed at lower doses. When the androgen-independent LAPC-4 tumors that did develop were subjected to the same serial dilution analysis, as few as 100 cells (*versus* 5×10^4 cells) was sufficient to form tumors in female mice (data not shown), demonstrating a 500-fold enrichment for androgen-independent cells by passage in an androgen-depleted environment. For LAPC-9, 13 of 88 (15%) female mice developed tumors at a pool size of 5×10^5, and no tumors were observed at lower cell doses. The fact that some but not all female animals develop tumors at defined cell doses argues against the hypothesis that androgen independence results from adaptation of a population of androgen-dependent cells to an androgen-depleted environment. Rather, the data support the presence of a small number of cells that are androgen independent or have the capacity to become androgen independent in some, but not all, pools of androgen-dependent cells. If these cells preexist, we estimate their frequency to be

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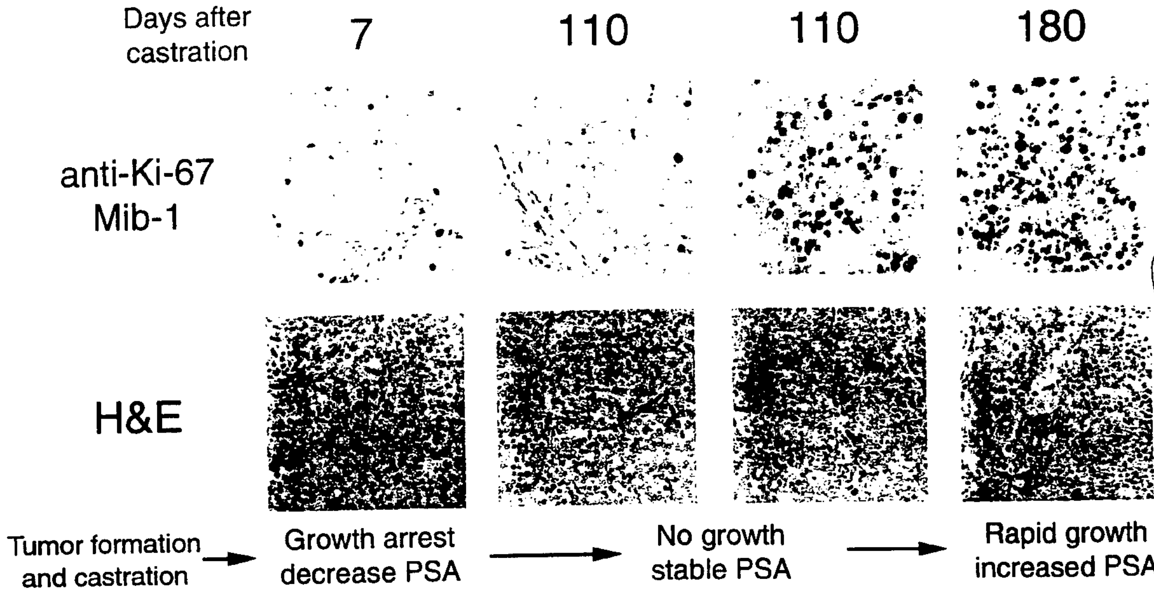

Fig. 4. Histological analysis of LAPC9 tumors after long-term castration. LAPC-9 AD cells were harvested and implanted as described in "Materials and Methods." Cells were implanted into intact male mice, and tumors were allowed to grow to ~0.5 cm^3 when the mice were castrated. Tumors were harvested at the time points indicated after castration and prepared for staining as described. Photomicrographs show staining results using the anti-Ki-67 antibody Mib-1 at 7, 110, and 180 days after castration. Two sections from the 110-day time point are shown to make the point that only restricted areas of focal proliferation can be detected at this time point. H&E staining is shown below.

about 1 per 10^5 for LAPC-4 and 1 per 10^6 for LAPC-9, which is consistent with the different latencies for LAPC-4 and LAPC-9 to develop androgen-independent tumor outgrowth after trocar implantation (Table 1). Alternatively, if these cells develop as a consequence of secondary genetic events that allow androgen-independent growth, the frequencies of 1 per 10^5-10^6 could reflect different mutation rates in LAPC-4 *versus* LAPC-9 because of genomic instability.

Histological Evidence for Outgrowth of Androgen-independent Subclones. One prediction of the hypothesis that androgen-independent cells are clonally derived from androgen-dependent cancers is that these cells should proliferate despite androgen ablation therapy. Because these cells represent such a small fraction of the androgen-dependent tumor, it would be difficult to visualize these cells using an immunohistochemical marker for cell proliferation unless the androgen-independent clone was allowed to expand to a detectable size. We tested this possibility by establishing androgen-dependent LAPC-9 tumors in intact males, castrating the mice, and examining serial histological sections of LAPC-9 tumors at time points before (day 110) and during (day 180) the outgrowth of androgen-independent tumors for evidence of proliferation using Ki-67. In this experiment, no animals were given supplemental testosterone after castration. At early time points (day 7), no foci of Ki-67-positive cells were observed, as seen previously in Fig. 2. However, at day 110, we observed small clusters of Ki-67-positive cells in a background of low-level, sporadic Ki-67-positive staining (Fig. 4, compare *panels 2* and *3* at day 110), indicating the presence of a focus of cell proliferation at a time prior to the outgrowth of an androgen-independent tumor. At 180 days, tumor size began to increase, indicating that an androgen-independent tumor had developed, and the majority of cells stained positive for Ki-67. These results provide histological evidence that androgen independence occurs by clonal outgrowth of a small number of androgen-independent cells.

DISCUSSION

Androgen plays a pivotal role in regulating the growth and differentiation of normal and malignant prostate epithelial cells. Although androgen ablation therapy produces dramatic clinical responses in prostate cancers, this treatment is palliative because androgen-independent or hormone-refractory tumors eventually regrow (35). The mechanism for this progression to androgen independence is unclear. In this report, we have taken advantage of two human prostate cancer xenografts developed recently by our group, each of which expresses PSA and wild-type androgen receptor, to characterize this process in more detail at the cellular level. Our results suggest that androgen-independent progression occurs in two distinct stages (Fig. 5). At the time of initial diagnosis, a fraction of cells in a prostate cancer tumor are dependent on androgen for survival (Fig. 5, lightly stippled cells) and undergo apoptosis in response to androgen ablation therapy, similar to the secretory epithelial cells in normal prostate tissue. Clinical evidence for this conclusion has been well documented in studies of prostate cancer tissue from patients who receive neoadjuvant hormone ablation therapy prior to radical prostatectomy surgery (5, 36, 37). The first step in androgen-independent progression is a transition stage in which tumor cells remain androgen responsive yet no longer require androgen for survival (Fig. 5, striped cells). The second stage involves the outgrowth of a tumor that is independent of androgen for both growth and survival, as observed clinically with hormone-refractory cancers that progress despite androgen ablation therapy (Fig. 5, dark gray cells). Through serial dilution studies and fluctuation analysis of LAPC-4 and LAPC-9, we provide evidence that this second stage results from clonal expansion of a small number of androgen-independent cells.

The strongest evidence for the first stage of androgen independence is the demonstration that a small number of LAPC-9 cells injected into castrated animals can survive for 6 months or more and remain

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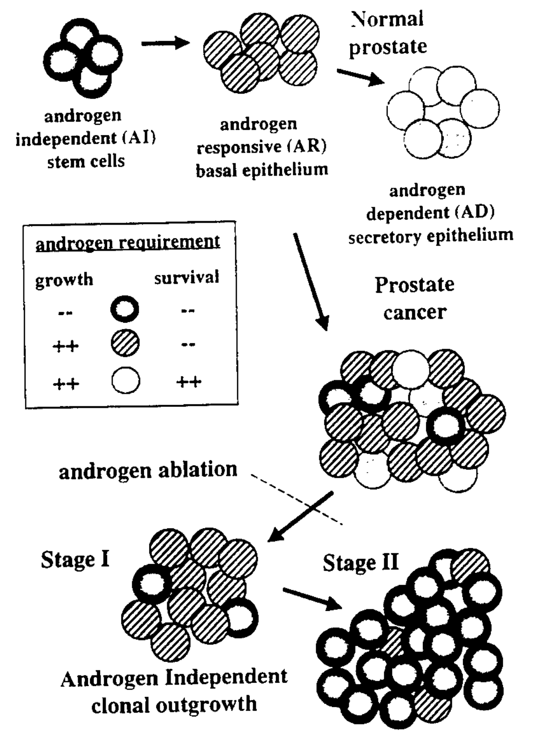

Fig. 5. Model for development of androgen-independent prostate cancer through two distinct stages. A two-step model for progression to androgen independence through clonal selection is shown. Three types of cells are postulated, which vary in their requirement for androgen as a growth and survival factor. Cells that require androgen for both growth and survival are represented by light stippling and correspond to the secretory epithelium in normal prostate. Cells that require androgen for growth but not survival [*androgen responsive (AR)*] are striped and may correspond to basal epithelial cells in normal prostate. Cells that do not require androgen for growth or survival [*androgen independent (AI)*] are represented by *dark gray*. It is unknown if a counterpart for these cells exists in the normal prostate, but it may correspond to the putative prostate stem cell. The first step in progression to androgen independence (stage I) is enrichment for androgen-responsive (*striped*) cells as a consequence of apoptotic death of the androgen-dependent (*light stippling*) cells after androgen ablation therapy. The second step (stage II) is clonal outgrowth of androgen-independent (*blue*) cells.

acutely responsive to androgen. This result defines a population of cells present in human prostate cancers that are dependent on androgen for growth but not survival. It also provides further support for the concept that androgen stimulates growth by acting directly on the prostate cancer cell rather than through stromal cells (38), because no supporting stroma or vasculature was allowed to develop. The presence of these stage I cells has immediate relevance for the clinical use of antiandrogen therapy, because it argues that these cells can be held in check but not eliminated by such a treatment strategy. It also provides an explanation for why androgen-dependent xenografts such as LAPC-4 (28), LAPC-9, LNCaP (39), and CWR22 (40) can be derived from patients with hormone-refractory cancer. These tumors presumably contain a mixture of growth-arrested, androgen-responsive tumor cells (stage I) in addition to androgen-independent cells (stage II) at the time of implantation into mice. In the androgenic environment of the intact male mouse, the androgen-responsive cells would gain a growth advantage and eventually develop into an androgen-dependent xenograft. Studies in rodent cancer models such as the Shionogi mouse mammary carcinoma (25, 26) and the Dunning rat prostate cancer model (20–22) have also described an androgen-responsive stage, providing further evidence for the presence of such cells in hormone-dependent human tumors. A critical next step is to identify the molecular basis for androgen-independent survival as opposed to androgen-independent growth. Because this phenotype shares many of the antiapoptotic features conferred by Bcl-2 overexpression in growth factor-dependent hematopoietic cells (41), it is logical that perturbations in this pathway may provide an explanation. Indeed, overexpression of Bcl-2 and Bcl-X_L has been reported in clinical prostate cancer specimens (42–46). It is also possible that molecular abnormalities linked previously to androgen-independent growth, such androgen receptor mutation or amplification (7–12), may play a role in androgen-independent survival.

The second stage of androgen-independent progression, in which tumors grow despite antiandrogen therapy, has been recognized for decades, but the cellular details have been unclear. Two findings from the xenograft studies presented here argue that this occurs through preferential expansion of a small number of androgen-independent cells present in the androgen-dependent xenografts under the selective pressure of androgen ablation therapy: (*a*) we can identify focal areas of cell proliferation that develop in LAPC-9 tumors that have undergone prolonged growth arrest in response to castration. We postulate that these foci undergo further clonal expansion and become hormone-refractory tumors; and (*b*) serial dilution studies demonstrate that androgen-independent cells account for 1 in $10^5/10^6$ cells in our androgen-dependent xenografts. This frequency could reflect the relative abundance of preexisting androgen-independent cells or the mutation rate for acquiring a genetic or epigenetic event that allows androgen-independent growth. A mutational frequency of 10^6 is consistent with the background of genomic instability known to exist in human prostate cancers (47–51) and shown previously to occur in the Dunning R-3327 rat prostatic adenocarcinoma system (22).

Our proposal that human prostate cancers progress to androgen independence through clonal evolution is similar in concept to conclusions about the process of cancer metastasis. The work of Fidler and colleagues (52, 53) has established that metastasis occurs by selection of a rare subpopulation of cells with metastatic potential from a heterogeneous starting population of tumor cells. Molecular evidence in support of this concept as applied to metastasis is now available from clinical studies comparing the frequency of cells harboring p53 mutations in metastatic prostate cancer lesions *versus* the primary tumor (54). Similar evidence for clonal expansion of hormone-refractory prostate cancer cells will require further progress in identifying the molecular lesions responsible for late-stage, androgen-independent disease.

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Mechanistic concepts in androgen-dependence of prostate cancer

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Key words: hormone-refractory, tyrosine kinase, Her-2/neu, androgen receptor, co-activator, co-repressor

Abstract

Androgen blockade is the mainstay of therapy in the clinical management of advanced prostate cancer. Recent progress on two fronts – the development of newer xenograft and transgenic models and a greater understanding of nuclear receptor signaling – has provided new insight into mechanisms of androgen-dependence in prostate cancer. This review centers on the concept that perturbations in androgen receptor signaling are likely to occur early in prostate cancer and play a critical role in progression to end stage hormone-refractory disease.

Role of androgen in the normal prostate gland

Androgen plays a critical role in regulating the growth, differentiation and survival of epithelial cells in the normal prostate (Figure 1). In humans the most compelling evidence comes from studies of men with androgen deficiency states such as eunuchs or individuals with hereditary 5-α reductase deficiency who have underdeveloped prostates as a consequence of low circulating testosterone [1,2]. Rodent studies have led to the concept that androgen functions as a survival factor in the adult prostate. Following castration the prostate gland of adult male rats involutes due to massive apoptosis of the secretory epithelial cells [3–5]. Histologically the secretory epithelial layer is lost but the basal epithelial cells and basement membrane remain intact, suggesting that the secretory epithelial cells require androgen for survival whereas the basal epithelial cells do not. When testosterone is replaced in castrated males, the prostate gland can be reconstituted to its normal weight through the formation of new ductules that originate from a subset of cells that line the remnant ducts. These studies demonstrate that stem-like cells (presumably basal epithelial cells) exist in the normal prostate and that they do not require androgen for survival but proliferate in response to androgen exposure.

The most straightforward model to explain these findings is a direct effect of androgen on epithelial cells. However, there is strong evidence that androgen-responsive prostate stromal cells mediate some of these effects through paracrine production of peptide hormones that act on the basal epithelium. Reconstitution experiments show that mesenchymal cells which give rise to stroma can induce the differentiation of fetal urogenital sinus tissue into prostate epithelial cells when co-transplanted under the kidney capsule of rats [6,7]. Androgen is hypothesized to function in this process by acting on a subset of stromal cells that express the androgen receptor and respond to androgen by secreting peptide hormones such as keratinocyte growth factor (KGF), insulin-like growth factor-1 (IGF-1), fibroblast growth factor (FGF) and epidermal growth factor (EGF) [8,9]. Through effects on the basal epithelial cells, these peptide hormones are postulated to function as inducers of prostate differentiation, including transcriptional activation of the androgen receptor. While the mechanistic details need further clarification, it seems clear that androgen affects the prostate epithelium through a complex set of direct and indirect mechanisms that produce distinct outcomes – growth versus survival – in distinct epithelial cell types.

Classifying prostate cancers on the basis of androgen-dependence – distinguishing growth from survival

The terms androgen-dependent or androgen-independent are widely used to classify prostate cancers

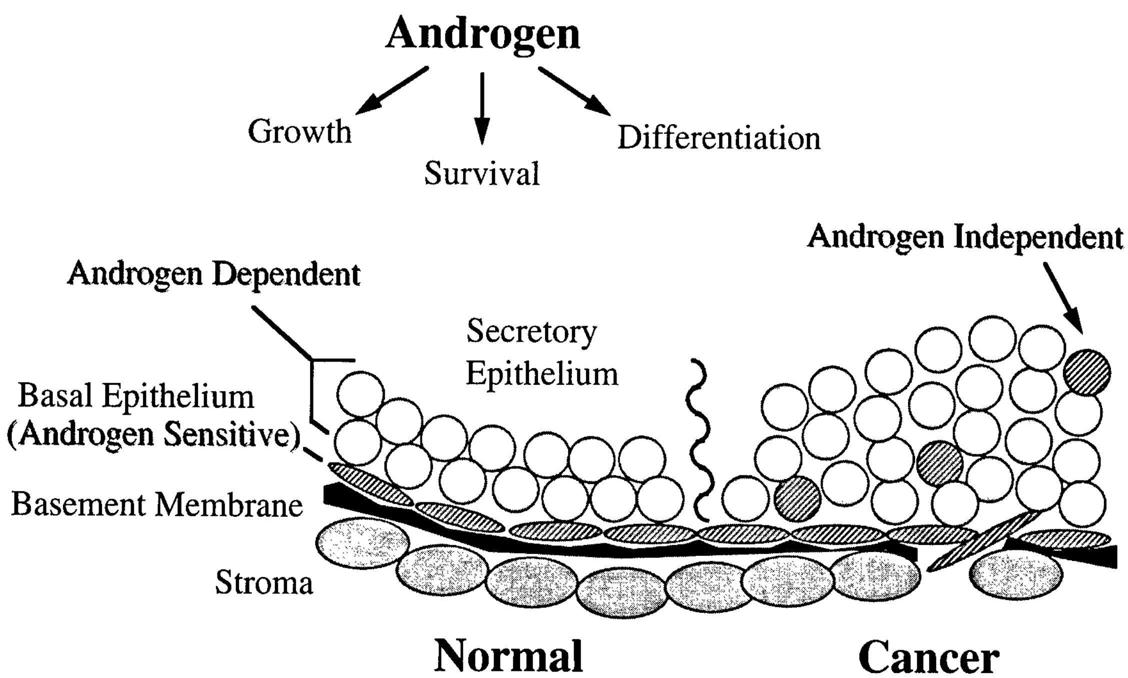

Figure 1. Model for prostate cancer progression based on normal prostate histology. The histologic features of the normal prostate gland (left) include stromal cells, basement membrane, basal epithelial cells and secretory cells. The secretory epithelial cells express PSA and are dependent on androgen for survival. The basal epithelial cells do not express PSA, do not require androgen for survival, but respond to androgen post-castration. Prostate cancer cells (right) express secretory cell differentiation markers such as PSA and are dependent on androgen for growth but not survival. Under the selective pressure of androgen ablation therapy, a subclone of cancer cells emerges which is no longer dependent on androgen for growth or survival. This subclone is responsible for hormone-refractory disease.

based on the response to androgen ablation therapy. Generally androgen-dependent tumors either regress or stop growing when anti-androgen therapy is initiated, and serum levels of the androgen-regulated gene prostate specific antigen (PSA) decline. Cancers which relapse despite androgen ablation therapy or which fail to respond to initial anti-androgen therapy are termed androgen-independent or hormone-refractory. While clinically useful, these designations fail to distinguish between effects of androgen on growth versus survival. Recent evidence indicates that these two effects, which are distinct in basal versus secretory epithelial cells of normal prostate, also appear to be distinct in prostate cancer cells. Experiments from our laboratory show that LAPC-9 prostate cancer xenograft cells are dependent on androgen for growth because serum PSA levels fall and tumors stop growing in mice treated with castration. However, LAPC-9 cells do not require androgen for survival because very few cells undergo apoptosis after castration, and these cells rapidly resume a normal growth rate when re-exposed to androgen, even after extremely long time intervals. Analysis of clinical material in patients treated with androgen ablation therapy is consistent with the model that prostate cancer cells no longer require androgen for survival despite remaining androgen-dependent for growth. The primary histologic consequence of androgen ablation in patients is growth arrest rather than apoptosis of tumor cells [10]. The collective evidence from xenograft models and clinical material indicates that prostate cancers overcome the need for androgen as a survival factor early in the evolution of the cancer. The molecular event responsible for this change remains to be defined.

One issue raised by these observations is whether early androgen ablation therapy might prevent this escape from androgen-dependent survival. While this concept is extremely controversial clinically, studies in transgenic mice which develop prostate cancer as a consequence of SV40 T antigen expression show that early castration can cure 20 percent of mice [11]. However, those tumors that do develop have a poorly differentiated histology and, presumably, a more aggressive phenotype. These findings suggest

that androgen also functions as a differentiation factor and that early androgen ablation therapy may force the outgrowth of less differentiated cancer cells.

Hormone-refractory prostate cancers retain constitutive androgen receptor signaling

Tumors which relapse following androgen ablation therapy are termed hormone refractory. One early hypothesis is that this late stage of prostate cancer is a consequence of loss of androgen receptor expression, analogous to poor-prognosis, estrogen receptor-negative breast cancer. Although several prostate cancer models fail to express androgen receptor [12–15], studies of patient material show that nearly all cancers retain androgen receptor expression regardless of clinical stage or hormone status [16–19]. Indeed, the fact that the majority of hormone-refractory cancers express the androgen-responsive PSA gene indicates that the androgen receptor signaling pathway is functional. Current evidence favors a model where additional molecular changes occur during progression to the hormone-refractory state that allow the androgen receptor pathway to be active in the absence of ligand or in the presence of androgen receptor antagonists.

Androgen receptor abnormalities

One mechanism to activate the androgen receptor pathway is through alterations in the androgen receptor itself. Several studies examining this question report androgen receptor mutations in 10–30 percent of patients with hormone-refractory disease [20–23]. When present these mutations tend to occur in patients treated with androgen receptor-antagonists such as flutamide or casodex rather than patients treated solely with castration or leutinizing hormone releasing hormone (LHRH) agonists. This association with drugs which function as competitive antagonists of the androgen receptor argues that these mutations occur under extreme selective pressure and must provide some competitive advantage. One of the best studied examples is mutation of codon 868 from threonine to alanine, which alters steroid binding characteristics and causes the receptor to be activated by non-androgenic steroids [24]. Other mutations in the hormone-binding domain can produce related effects, including mistaken recognition of an antagonist as an agonist. This latter example provides a potential explanation for flutamide withdrawal syndrome, the clinical term applied to cancers progressing on anti-androgen therapy which respond, paradoxically, to withdrawal of the androgen receptor antagonist (reviewed in [16]). Another androgen receptor abnormality, amplification of the wild-type receptor locus on the X chromosome, has also been described in about 20 percent of patients with hormone-refractory disease [19]. Although androgen receptor abnormalities are unlikely to account for the majority of hormone-refractory prostate cancers, they do illustrate the central role of altered androgen receptor function in late stage disease.

Intracellular signaling pathways that influence androgen receptor function

In addition to alterations in the androgen receptor itself, it clear that the androgen receptor pathway can be affected through other mechanisms (Figure 2). The peptide hormones IGF-1, KGF and EGF, which serve as ligands for receptor tyrosine kinases and activate downstream intracellular kinase cascades, also activate the androgen receptor pathway [25]. Each of these growth factors can activate transcription from an androgen-responsive reporter construct in the absence of ligand or synergistically in conjunction with androgens. The fact that this activation can be blocked by the androgen receptor antagonist casodex indicates that these effects require the androgen receptor. Other stimuli which do not function through receptor tyrosine kinases can also activate the androgen receptor pathway. These include forskolin, which increases cellular cAMP through protein kinase A [26], and interleukin-6, which is frequently elevated in the sera of patients with prostate cancer [27]. Conversely, a protein kinase A-specific inhibitor decreased activation of the androgen receptor by natural ligands, forskolin or interleukin-6 [26,27]. These findings show that activation of several tyrosine or serine/threonine kinase pathways can modulate androgen receptor activity. More work is needed to determine if these effects are all mediated through a common final pathway or whether each stimulus independently activates the androgen receptor.

It will be important to define the clinical significance of these findings with reference to prostate cancer. One hypothesis is that excess levels of peptide hormones such as IGF-1, which are associated with increased prostate cancer risk [28], produce alterations in androgen receptor function that affect epithelial cell growth in the prostate. Indeed, autocrine production of these

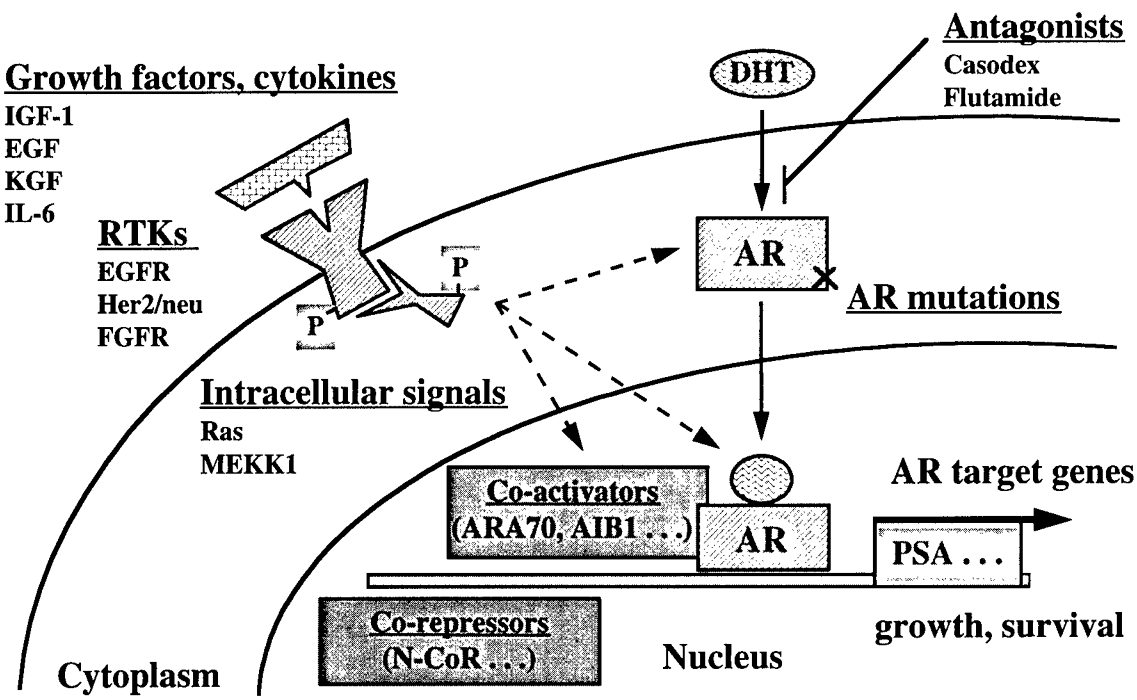

Figure 2. Mechanisms of ligand-independent androgen receptor activation. Activation of the androgen receptor signaling pathway is a central theme in hormone-refractory prostate cancer. This activation can occur through mutations in the androgen receptor which alter specificity for ligands or through upregulation of non-steroid receptor signaling pathways that activate androgen receptor signaling through unknown mechanisms. Inhibitors of these pathways may have therapeutic utility in hormone-refractory prostate cancer.

peptide hormones has been described in some prostate cancers [29–35]. While compelling, these observations are unlikely to explain disease progression in patients treated with androgen receptor antagonists because the effects of these peptide hormones on androgen receptor function can be blocked by the androgen receptor antagonist casodex.

Recent data from our laboratory suggest that excess activation of certain EGF receptor family members may provide an explanation for some cases of hormone refractory cancer. In previous work we reported that the LAPC-4 xenograft can progress from androgen-dependence to androgen-independence in response to castration in the absence of mutations in the androgen receptor [36]. In a survey of tyrosine kinases whose expression might change during this evolution to a hormone-refractory state, we noted that HER-2/neu, an EGF receptor family member, was consistently overexpressed in androgen-independent sublines. In addition, forced overexpression of HER-2/neu in androgen-dependent cells conferred androgen independent growth and activated transcription from the PSA promoter in the absence of ligand. Notably, the activation of downstream androgen-dependent genes by HER-2/neu occurs synergistically with androgen and requires the androgen receptor. Unlike IGF-1 or KGF, the effects of HER-2/neu on PSA transcription cannot be blocked by casodex, suggesting that HER-2/neu affects the androgen receptor pathway at a point downstream from the ligand binding site. The fact that the HER-2/neu effects are casodex-independent is consistent with a clinically relevant role in hormone refractory cancer. In conjunction with studies of clinical material that report HER-2/neu overexpression in some prostate cancers [37–42], these findings argue that therapeutic strategies that target HER-2/neu may have utility. Clinical trials with the anti-HER-2/neu monoclonal antibody Herceptin, recently approved for use in breast cancer, are currently being planned in prostate cancer.

Alterations in the expression of genes which regulate apoptosis have also been implicated in hormone-refractory prostate cancer. Bcl-2, an inhibitor of apoptosis in many types of tumors, is expressed in a subset of the basal epithelial cells but not in the secretory epithelial cells of the normal prostate [43,44].

However, many late stage, androgen-independent prostate cancers regain expression of Bcl-2 [45]. This phenomenon is also observed in the LuCaP23 xenograft model. Androgen-dependent sublines do not express Bcl-2, but Bcl-2 positive cells are detected after castration, consistent with clonal expansion of an androgen-independent population [46]. In the LNCaP model Bcl-2 can partially replace the need for androgen because forced overexpression of Bcl-2 allowed faster recovery of growth post-castration [47]. While these experiments provide functional evidence that Bcl-2 can overcome the androgen requirement of LNCaP cells, it is unclear if Bcl-2 functions through activation of the androgen receptor pathway or through downstream blockade of apoptotic pathways that would normally be activated by androgen withdrawal. Indeed, inhibition of caspases by CrmA also blocks apoptosis induced by androgen ablation [48].

Nuclear receptor co-activators and co-repressors

Recent studies in the nuclear receptor field have identified families of proteins known as co-activators and co-repressors which modulate the function of transcription factors. Although the role of these proteins in androgen receptor function is untested, the fact that many are broadly involved in regulating a range of transcription factors argues for a potential role in androgen receptor function. The most compelling example is a novel gene called AIB1, a member of the SRC-1 family of nuclear receptor co-activators amplified in breast and ovarian cancer [49]. AIB1 appears to function as a co-activator since it binds to the estrogen receptor in a ligand-dependent fashion and enhances estrogen-dependent gene transcription. Because AIB1 contains the highly conserved nuclear receptor interaction motif LXXLL, it is likely that additional nuclear receptors can function as AIB1 partners. In addition to these general co-activators, androgen receptor-specific regulators may exist. ARA70, which was isolated in a yeast 2-hybrid screen for proteins that bind the androgen receptor, may be one such example [50]. Expression of ARA70 specifically enhances transcription of androgen-responsive genes and can alter the ligand specificity of the androgen receptor such that antagonists function as agonists, analogous to the effects of certain androgen receptor mutations [51].

In addition to an increase in the level of co-activators, hormone receptor function can be enhanced through downregulation of co-repressors. The breast cancer example is most instructive where a decrease in the level of the co-repressor N-CoR is correlated with acquisition of resistance to the estrogen receptor antagonist tamoxifen in a xenograft model [52]. In the presence of tamoxifen, N-CoR binds to the estrogen receptor and blocks transcription from estrogen-responsive promoters. Reduced levels of N-CoR presumably allow estrogen-dependent gene expression (and tumor progression) to occur despite tamoxifen. The action of peptide growth factors such as EGF on hormone receptors might also be explained through effects on N-CoR since stimulation of breast cancer cells with EGF can block the tamoxifen-dependent interaction of N-CoR and estrogen receptor. It will be important to determine if AIB1, N-CoR or related proteins also play a role in prostate cancer.

Summary

Current studies show that most prostate cancers express androgen receptor at all stages of disease, including hormone refractory cancer. Taken together with the fact that most tumors retain expression of androgen-dependent genes, it appears that perturbations of the androgen receptor pathway play a central role in prostate cancer. Recent xenograft and transgenic models suggest that, early in their evolution, prostate cancers escape the requirement for androgen as a survival factor while retaining dependence on androgen as a growth factor. Progression to hormone-refractory prostate cancer appears to involve an additional molecular event that allows cells to overcome the dependence on androgen for growth. Molecular studies indicate that androgen receptor function can be modulated through mutations in the receptor itself or through alterations in non-steroid ligands, kinases and co-activators or co-repressors which influence the androgen receptor pathway. It is becoming increasingly clear that further mechanistic understanding of the androgen receptor pathway will provide new insight into prostate cancer treatment. One immediate possibility is that inhibitors of tyrosine kinase signaling pathways that influence androgen receptor function may be useful in hormone refractory prostate cancer.

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